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FUMARATE HYDRATASE AND SUCCINATE DEHYDROGENASE IN NEOPLASIA

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Academic Dissertation

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To Rita and to my family

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"…the process of experimental science does not consist in explaining the unknown by the known, as in certain mathematical proofs. It aims, on the contrary, to give an account of what is observed by the properties of what is imagined. To explain the visible by the invisible. And it is through the evolution of the invisible, through an appeal to new hidden structures, with hypothetical properties, that science proceeds."

-François Jacob

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the 4 original contributions listed below. They will be referred to by Roman numerals I-IV in the text.

I. Sakari Vanharanta*, Mary Buchta*, Sarah R. McWhinney*, Sanna K. Virta*, Mariola Peçzkowska, Carl D. Morrison, Rainer Lehtonen, Andrzej Januszewicz, Heikki Järvinen, Matti Juhola, Jukka-Pekka Mecklin, Eero Pukkala, Riitta Herva, Maija Kiuru, Nina N. Nupponen, Lauri A. Aaltonen, Hartmut P. H. Neumann and Charis Eng. Early-onset renal cell carcinoma as a novel extraparaganglial component of SDHB-associated heritable paraganglioma. *American Journal of Human Genetics* (2004) 74, 153-159

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IV. Sakari Vanharanta*, Noel C. Wortham*, Cordelia Langford, Mona El-Bahwary, Zephne van der Spuy, Jari Sjöberg, Rainer Lehtonen, Auli Karhu, Ian P.M. Tomlinson and Lauri A. Aaltonen. Definition of a Minimal Region of Deletion of Chromosome 7q in Uterine Leiomyomas by Tiling-Path Microarray CGH and Screening of Known Genes in This Region, submitted

*Equal contribution

ABBREVIATIONS

7QD	fibroids showing allelic imbalance on chromosome 7q
AATF	apoptosis antagonizing transcription factor
aCGH	microarray comparative genomic hybridisation
ACTB	beta-actin
AI	allelic imbalance
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)
APC	adenomatosis polyposis coli
ATM	ataxia telangiectasia mutated
ATP	adenosine trisphosphate
BAC	bacterial artificial chromosome
BCL6	B-cell CLL/lymphoma 6
BHD	Birt-Hogg-Dubé
bp	base pair
CCND1	cyclin D1
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
cDNA	complementary DNA
CGH	comparative genomic hybridisation
COG5	component of oligomeric golgi complex 5
COX6C	cytochrome c oxidase subunit VIc
CRC	colorectal caner
cRNA	complementary RNA
DAPK1	death-associated protein kinase 1
DNA	deoxyribonucleic acid
DOP-PCR	degenerate oligonucleotide-primed PCR
dNTP	deoxynucleotide triphosphate
EPO	erythropoietin
FDR	false discovery rate
FGEA	functional group enrichment analysis
FH	fumarate hydratase, fumarase
FMTC	familial medullary thyroid carcinoma
GADD45B	growth arrest and DNA-damage-inducible beta
GLUT1	glucose transporter 1
GO	Gene Ontology
GSEA	gene set enrichment analysis
HE	haematoxylin-eosin
HIF	hypoxia-inducible factor
HIF1α	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HIF1β	hypoxia-inducible factor 1, beta subunit
HIF2α	hypoxia-inducible factor 2, alpha subunit
HLRCC	hereditary leiomyomatosis and renal cell cancer
HMGA1	high mobility group AT-hook 1
HMGA2	high mobility group AT-hook 2
HNP	head and neck paraganglioma
HPGL	hereditary paraganglioma
HOXA5	homeo box A5
HPT-JT	hyperparathyroidism-jaw tumour
KRAS	v-KI-RAS2 kirsten rat sarcoma 2 viral oncogene homolog

LDHA	lactate dehydrogenase A
LOH	loss of heterozygosity
MAS	microarray suite
Mb	mega base pairs
MCUL	multiple cutaneous and uterine leiomyomata
MEN2	multiple endocrine neoplasia type 2
MEN2A	multiple endocrine neoplasia type 2A
MEN2B	multiple endocrine neoplasia type 2B
MET	met proto-oncogene (hepatocyte growth factor receptor)
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1
mTOR	mammalian target of rapamycin
N7Q	fibroid not showing allelic imbalance on chromosome 7q (normal 7q)
NF1	neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)
p53	tumour protein 53
PCA	principal component analysis
PCR	polymerase chain reaction
PDE8B	phosphodiesterase 8B
PDGF	platelet-derived growth factor
PFVD	polyvinylidene fluoride
PGL	paraganglioma
PM/MM	perfect match/mismatch
PRKCB1	protein kinase C beta 1
pVHL	product of the VHL gene
RAD51L1	RAD51-like 1
RARRES3	retinoic acid receptor responder 3
RAS	Harvey and Kirsten sarcoma virus gene
RB	retinoblastoma gene
RCC	renal cell cancer
RET	ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
RINT-1	Rad50-interacting protein 1
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
SAM	significance analysis of microarray
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein
SDHB	succinate dehydrogenase complex, subunit B, iron sulfur
SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein
SDHD	succinate dehydrogenase complex, subunit D, integral membrane protein
SMAD4	SMA- and MAD-related protein 4
SNP	single nucleotide polymorphism
Taq	*Thermus aquaticus*
TBP	TATA-box binding protein
TCAC	tricarboxylic acid cycle
TFAP2C	transcription factor AP-2 gamma
TGFBR2	transforming growth factor β receptor II

TKT	transketolase (Wernicke-Korsakoff syndrome)
TSC1	tuberous sclerosis 1
TSC2	tuberous sclerosis 2
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau (VHL) disease
XPB	xeroderma pigmentosum, complementation group B

ABSTRACT

Germline mutations in *fumarate hydratase* (*FH*) cause hereditary leiomyomatosis and renal cell cancer (HLRCC). FH is a nuclear encoded enzyme which functions in the Krebs tricarboxylic acid cycle, and homozygous mutation in *FH* lead to severe developmental defects. Both uterine and cutaneous leiomyomas are components of the HLRCC phenotype. Most of these tumours show loss of the wild-type allele and, also, the mutations reduce FH enzyme activity, which indicate that *FH* is a tumour suppressor gene. The renal cell cancers associated with HLRCC are of rare papillary type 2 histology. Other genes involved in the Krebs cycle, which are also implicated in neoplasia are 3 of the 4 subunits encoding succinate dehydrogenase (SDH); mutations in *SHDB*, *SDHC*, and *SDHD* predispose to paraganglioma and phaeochromocytoma.

Although uterine leiomyomas (or fibroids) are very common, the estimations of affected women ranging from 25% to 77%, not much is known about their genetic background. Cytogenetic studies have revealed that rearrangements involving chromosomes 6, 7, 12 and 14 are most commonly seen in fibroids. Deletions on the long arm of chromosome 7 have been reported to be involved in about 17 to 34 % of leiomyomas and the small commonly deleted region on 7q22 suggests that there might be an underlying tumour suppressor gene in that region.

The purpose of this study was to investigate the genetic mechanisms behind the development of tumours associated with HLRCC, both renal cell cancer and uterine fibroids. Firstly, a database search at the Finnish cancer registry was conducted in order to identify new families with early-onset RCC and to test if the family history was compatible with HLRCC. Secondly, sporadic uterine fibroids were tested for deletions on 7q in order to define the minimal deleted 7q-region, followed by mutation analysis of the candidate genes. Thirdly, oligonucleotide chips were utilised to study the global gene expression profiles of uterine fibroids in order to test whether 7q-deletions and *FH* mutations significantly affected fibroid biology.

In the screen for early-onset RCC, 214 families were identified. Subsequently, the pedigrees were constructed and clinical data obtained. One of the index cases (RCC at the age of 28) had a mother who had been diagnosed with a heart tumour, which in further investigation turned out to be a paraganglioma. This lead to an

alternative hypothesis that SDH, instead of FH, could be involved. *SDHA*, *SDHB*, *SDHC* and *SDHD* were sequenced from these individuals; a germline *SDHB* R27X mutation was detected with loss of the wild-type allele in both tumours. These results suggest that germline mutations in the *SDHB* gene predispose to early-onset RCC establishing a novel form of hereditary RCC. This has immediate clinical implications in the surveillance of patients suffering from early-onset RCC and phaeochromocytoma/paraganglioma.

For the studies on sporadic uterine fibroids, a set of 166 fibroids from 51 individuals were collected. The 7q LOH mapping defined a commonly deleted region of about 3.2 mega bases (Mb) in 11 of the 166 tumours. The deletion was consistent with previously reported allelotyping studies of leiomyomas and it therefore suggested the presence of a tumour suppressor gene in the deleted region. Furthermore, the high-resolution aCGH-chip analysis refined the deleted region to only 2.79 Mb. When combined with previous data, the commonly deleted region was only 2.3 Mb. The mutation screening of the known genes within the commonly deleted region did not reveal pathogenic mutations, however.

The expression microarray analysis revealed that FH-deficient fibroids, both sporadic and familial, had their distinct gene expression profile as they formed their own group in the unsupervised clustering. On the other hand, the presence or absence of 7q-deletions did not significantly alter the global gene expression pattern of fibroids, suggesting that these two groups do not have different biological backgrounds. Multiple differentially expressed genes were identified between *FH* wild-type and *FH*-mutant fibroids, and the most significant increase was seen in the expression of carbohydrate metabolism-related and hypoxia inducible factor (HIF) target genes.

1 PROLOGUE

The background of this particular work was laid when a person from a family with apparent familial clustering of renal cell cancer (RCC) was referred to genetic counselling some time during the mid-1990s. Instead of just adding the files to the pile of previously uncharacterised, but still probable cases of inherited renal cancer, the clinician, Dr. Outi Vierimaa, dug into the family's medical history thoroughly. This lead to the recognition of other clinical features that seemed to belong to the phenotype, namely cutaneous and uterine leiomyomas. The next step was to contact Professor Lauri A. Aaltonen, who had a lot of experience in mapping the genes responsible for hereditary cancer. The clinical observation had led to a scientific hypothesis that was to be rigorously tested in subsequent experimental work in the laboratory. With enormous amounts of time and effort, the gene responsible for the new syndrome was first mapped to a particular region of the genome, and subsequently cloned. The molecular background of a new tumour predisposition syndrome, hereditary leiomyomatosis and renal cell cancer (HLRCC), had been elucidated.

This process that lead from the original clinical observation into the scientific success story is depicted in Figure 1. This scheme of thought is not complete, nor does it take into account much of the literature of the philosophy of science, but it still has been important during this work. It owes a great deal to the ideas of Karl R. Popper and his theory of scientific discovery. Whether the important thing in the process is the question, as François Jacob has put it, or the hypothesis, in the spirit of Popper, most theoretical views on practical everyday science are oversimplified. Still, it is important that everyone involved in the process of scientific research understands, at least in some personal

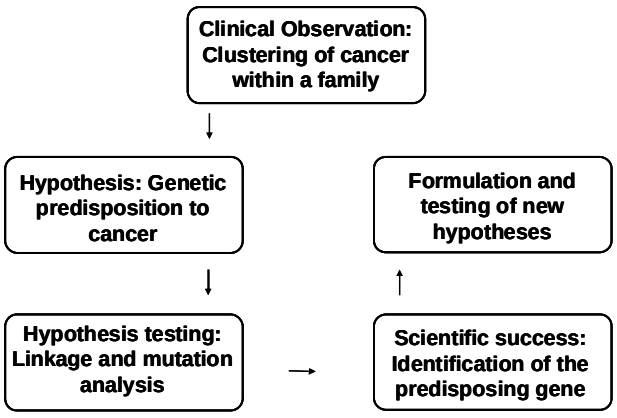

Figure 1.

way, logic behind their work. If not, the justification of the whole scientific research is wiped away: There is no science.

The work described here belongs to the second generation of experiments following the first scientific success after the initial clinical observation. If the gene would not have been identified, which indeed is the case rather often, the whole process would have stopped. On the contrary, the identification of the gene has lead to a huge number of different lines of research that all are linked through the common origin, the *FH* gene. This work is one small part of the research that has followed the discovery of the molecular background of HLRCC.

2 REVIEW OF THE LITERATURE

2.1 Some landmarks in tumour genetics

Although some opportunistic views still persist, cancer is generally thought as being a disease of genes (Vogelstein and Kinzler 2002). The first recognition of chromosome abnormalities as a cause of abnormal growth came through the studies of Theodor Boveri (1862-1915). He used sea urchin eggs to analyse the consequences of chromosome rearrangement on cell division, which lead him to postulate that similar aberrations could be the underlying cause of human malignancies (Balmain 2001, Wunderlich 2002). After this initial discovery, Boveri went on to make several predictions on the nature of human cancer that were to be supported by experimental results only decades later. For example, he foresaw the existence of cell-cycle checkpoints, tumour suppressor genes, oncogenes, the progression of tumours from benign lesions into malignant cancer, the clonality of tumours, cancer predisposition through inheritance of chromosomes, and the inheritance of some weak chromosomes that would, when homozygous, lead to high-penetrance cancer predisposition. These theoretical ideas were presented in his publication "*Zur Frage der Entstehung Maligner Tumoren*" in 1914.

The development of molecular biology through studies that showed that the substance transmitting inheritance was deoxyribonucleic acid (DNA) (Avery et al. 1944), the subsequent discovery of DNA structure (Watson and Crick 1953a, and 1953b), and the unravelling of the genetic code (Crick et al. 1961) lay further emphasis on the importance of the genetic component on the development of human disease. This was also reflected in the field of cancer research and lead to several landmark discoveries which still make the foundation of our current understanding of cancer.

Firstly, the development of cytogenetic techniques enabled the detection of chromosomal abnormalities in specific types of cancer, the first example of which is the Philadelphia chromosome described in chronic myeloid leukaemia in 1960 (Nowell and Hungerford). The specific chromosomal aberration underlying the Philadelphia chromosome was identified a decade later (Rowley 1973), after which the characterisation of the gene product resulting from the chromosomal abnormality became possible (Heisterkamp et al. 1983, de Klein et al. 1982). Later, several other

recurrent chromosomal aberrations have been detected in various cancers, and large scale somatic alterations are thought to be an important characteristic of several human tumours (Lengauer et al. 1998).

The second landmark in the history of cancer genetics was the discovery of oncogenes. The preliminary findings that cell-free mediators could transfer tumours from one chicken to another were made by Rous (1910, and 1911). His work was followed by a number of studies on the Rous Sarcoma Virus (RSV), and similar viruses were thought to be responsible for most, if not all cancers (Huebner and Todaro 1969). However, the discovery that the transforming agent in these viruses was an RNA sequence (Duesberg and Vogt 1970), and that this sequence had homologues in normal avian DNA (Stehelin et al. 1976) suggested that the viruses had obtained their transforming capability by "kidnapping" normal avian genes. Thus, it was proposed that tumours could arise without the involvement of viruses. First results supporting this hypothesis came when it was shown that DNA from chemically mutagenised transformed mouse cells could transform normal cells (Shih et al. 1979). Transforming sequences were subsequently extracted from human cancer as well, and soon the gene responsible for the process was identified as a cellular homologue of the Harvey and Kirsten sarcoma virus gene, *ras* (Parada et al. 1982). Initially the difference between the normal *c-Ha-RAS1* and the transforming counterpart from the carcinoma was not clear. Soon, however, the responsible amino acid change in the carcinoma cell line was identified at codon 12 (Reddy et al. 1982, Tabin et al. 1982), and subsequent research has shown that this mutation alters the protein as being constitutively active. Thus, molecular evidence for the presence of dominantly acting oncogenes had been produced and, subsequently, several dominantly acting cancer genes have been identified, some of which can also cause hereditary cancer (Futreal et al. 2004).

The final landmark has been the discovery of tumour suppressor genes. The familial clustering of cancer had been obvious from clinical observations for a long time, but the first theory that allowed the molecular testing of specific hypothesis on familial cancer came from the studies by Knudson (1971) on familial and sporadic retinoblastoma. He proposed a model according to which cancer arose through two hits on a cancer gene. This theory predicted that a gene acting recessively at the cellular level, a tumour suppressor gene, needed either a germline mutation and a somatic mutation, or alternatively, two somatic mutations for tumour development.

After the appropriate methodology became available for molecular detection of particular parental alleles, Knudson's theory led to the chromosomal mapping (Cavenee et al. 1983) and cloning (Friend et al. 1986) of the first tumour suppressor gene, *RB*. Since then, several recessively acting cancer genes have been discovered and, currently, more recessive than dominant cancer genes are known (Futreal et al. 2004).

2.2 Defining cancer

2.2.1 The number of mutations in tumours

Although it is not entirely clear whether all cancers are actually genetic diseases, as some might, at least theoretically, develop solely due to epigenetic changes, the study of mutations in cancer has proven an excellent approach to unravel the biology of many tumours. Early studies that applied mathematical models on mortality rates and epidemiological data concluded that cancer could not result from a single genetic hit. The first estimations were that around 7 mutations were required (Armitage and Doll 1954, Nordling 1953), and further refinement of the models suggested that 2 mutations could be enough (Armitage and Doll 1957). The results by Knudson on familial and sporadic retinoblastoma further supported the multi-step cancer progression theory and provided a model that could be further experimentally tested on the molecular level (Knudson 1971). Some have argued that in order to develop a sufficient amount of mutations, a cancer has to develop a "mutator phenotype" (Loeb et al. 1974). This view has, however, been contradicted by others, who have shown that evolution at the somatic level and the Darwinian selection is more important, although some cancers may have increased mutation rates that could speed up the evolutionary process (Tomlinson and Bodmer 1999, Tomlinson et al. 1996).

At present, there are about 350 known genes that are mutated in cancer (Futreal et al. 2004) (http://www.sanger.ac.uk/genetics/CGP/Census/); they represent more than 1% of all human genes. Approximately 90% of the cancer genes show somatic mutations and, in about 20%, germline mutations have been observed. Of the known somatic mutations, 90% act dominantly at the cellular level, whereas of the germline mutations, 90% are recessive. An important source for mutational information in cancers has been derived from recent large-scale resequencing efforts, which have typically targeted a large group of genes in a particular type or types of

tumours (Bardelli et al. 2003, Davies et al. 2005, Hunter et al. 2006, Samuels et al. 2004, Stephens et al. 2005, Wang et al. 2004).

The mutations detected in tumours can be divided into different classes, those that are contributing to the tumour growth (drivers), and those that only reflect the mutational processes that have been active during the tumorigenesis, but that do not contribute to the tumorigenic process (passengers) (Stratton 2006). The number of driver mutations in a particular tumour is determined by the number of events required for tumorigenesis, and the pattern of these mutations reflects the mutational processes and selection. The number of passenger mutations, on the other hand, is determined by the number of mitoses during development, starting from the first mitosis in the embryogensis, and the pattern of these mutations solely reflects the mutational processes. As most mutations detected in an individual tumour are likely to be passengers, the problem is how to identify the drivers. The comparison of the amount of silent versus missense and nonsense mutations suggests, however, that drivers do exist.

A significant contribution to resequencing projects has come from the Sanger Cancer Genome Project that has focused on protein kinases. Their results indicate that there might be several different kinases contributing to tumorigenesis, making the identification of drivers difficult (Davies et al. 2005). Also, based on sequence data from the kinome, the amount of somatic mutations in an individual tumour has been estimated to range from less than 2000 to more than 100 000, resulting in 20 to 1000 amino acid changes (Stephens et al. 2005). In addition to leading to the identification of new genes mutated in different tumour types, these resequencing studies have provided significant insight into the biology of some of these tumours. For example, a screen for somatic mutations in breast cancers revealed a possible new mutator phenotype (Stephens et al. 2005), and a similar study on glioblastomas led to the identification of a new mechanism enabling tumours to survive and recur after chemotherapy (Hunter et al. 2006). Similar projects focusing solely on the cancer genome are likely to provide invaluable information of the biology of various human tumours in the near future.

2.2.2 Adenoma-carcinoma sequence

Although evidence supporting the idea of the clonal origin of cancer had emerged since the recognition of cancer being a genetic disease, it was not until 1976 that Nowell incorporated this with the idea of multistep tumour development (Nowell 1976). Direct experimental evidence in support of the clonal origin of tumours came from studies on the chromosome X inactivation pattern in colorectal cancer (CRC) (Fearon et al. 1987), and subsequently similar findings have been seen in other tumour types (Sidransky et al. 1992). Building on this, Fearon and Vogelstein constructed their famous genetic model for colorectal tumorigenesis (Fearon and Vogelstein 1990). They postulated, based on experimental evidence from studies on colorectal lesions of various stages, that the clonal evolution of tumours is indeed a multistep process in which the number of genetic hits, rather than the order in which they accumulate, is the key denominator of each neoplastic lesion. According to this model, changes in probably at least 5 different genes, the majority of which are tumour suppressors, were required for the malignant tumour to develop; fewer changes would be enough for adenomas. Based on evidence from studies on *p53*, a well established tumour suppressor gene, Fearon and Vogelstein argued that some tumour suppressors are not necessarily recessive on the cellular level, that is, already the loss of one allele would increase the susceptibility to tumorigenesis. It has also become evident that the different hits target more precisely different pathways, rather than different genes (Vogelstein and Kinzler 2004). This notion is based on genetic evidence from mutation screenings of CRCs, as it has been shown that mutations in certain genes, such as in *APC* and *β-catenin*, are mutually exclusive in individual tumours (Sparks et al. 1998). This is explained by the fact that both mutations are thought to have the same effect on the APC/ β-catenin/Tcf pathway and, therefore, acquiring mutations in both genes would not confer any further growth advantage. Although this adenoma-carcinoma sequence is rather well established in CRC, there is evidence that at least in tumours showing microsatellite instability, carcinomas would not develop from macroscopic adenomas (Tsao et al. 2000). It does not mean, however, that adenomas and carcinomas could not have a common progenitor, but it suggests that a slow process through which large adenomas gradually become more and more malignant is unlikely.

2.2.3 Gatekeepers, caretakers, and landscapers

Vogelstein has further developed his model on tumorigenesis by dividing the roles of individual genes into gatekeepers, caretakers, and landscapers (Kinzler and Vogelstein 1996, and 1998). Gatekeepers are genes that are directly involved in the regulation of cell birth or cell death. Mutations in these genes, such as *VHL*, *p53*, *RB*, and *APC*, are therefore essential for the tumorigenic process and, thus, normally function as "gatekeepers" to prevent uncontrolled growth. When the function of these genes is restored in cancer cells, tumour progression and growth is inhibited. Each cell-type has its own gatekeepers, and the tumour spectrum of different cancer predisposition syndromes varies. Also, same gatekeepers can act at different stages of tumour progression in different tissue-types. An example of this are mutations in *p53*, as they are present in about 80% of CRCs, but germline mutations in the same gene do not increase the risk of CRC (Kinzler and Vogelstein 1996).

Caretakers are genes that are involved in the maintenance of cell integrity, for example through DNA repair (Kinzler and Vogelstein 1997, and 1998). When these genes are inactivated, cellular processes become unstable therefore leading to increased rates of mistakes, such as mutations during DNA replication; the DNA repair genes, such as *XPB*, *ATM*, *MSH2*, and *MLH1*, are the prototypes of caretakers. Instead of being directly involved in tumorigenesis, loss-of-function mutations in these genes facilitate the acquisition of multiple genetic hits, some of which might promote tumorigenesis. In the case of mismatch repair genes, this is seen as increased mutation rates of short tandem nucleotide repeats, a phenomenon referred to as microsatellite instability (Kinzler and Vogelstein 1996). However, as caretakers are not directly involved in cell proliferation, the restoration of these genes in tumour cells does not have an effect on tumour growth; only the subsequent tumour evolution might slow down.

Landscapers, on the other hand, are genes that govern the cellular landscape (Kinzler and Vogelstein 1998). These genes function at the tissue level and regulate cells other than those in which they are expressed. An example of these genes is *SMAD4*, inherited mutations of which predispose to multiple hamartomatous gastrointestinal polyps. The increased risk of cancer associated with *SMAD4* mutations is thought to arise through abnormal stromal cell compartment, which leads to defective intercellular signalling with the epithelium, thus facilitating epithelial

transformation (Kinzler and Vogelstein 1998). More direct evidence on the landscaper effect has emerged from studies on mouse models. For example, the inactivation of the *transforming growth factor β receptor II* in mouse fibroblasts (*TGFBR2*) has resulted in intraepithelial neoplasia of the prostate and invasive squamous cell carcinoma of the forestomach (Bhowmick et al. 2004).

2.2.4 Order of mutations

The idea that only the number of mutations is important, not the order or time-frame in which they are acquired, has been challenged. At least theoretically, it seems reasonable that some mutations that are important for a tumour would be deleterious if acquired at the wrong time. This is due to a strong intrinsic tendency for tumour suppression that is inherent in all metazoan cells (Lowe et al. 2004), and reflected, for example, in the fact that oncogenes can cause apoptosis (Evan et al. 1992), and that by inactivating the apoptotic machinery at the same time, tumorigenesis may proceed (Pelengaris et al. 2002). In such situations, rescuing normal apoptotic function by restoring the normal expression of a gene, for example the tumour suppressor *p53*, could be fatal to the tumour but leave normal cells intact (Lowe et al. 2004). A similar idea of manipulating cellular pathways in a way which is deleterious for cancer cells but would have no effect on normal cells comes from the concept of synthetic lethality (Kaelin 2005). Two genes are synthetically lethal if inactivation of either of them alone does not have an effect on the cell, but inactivating both of them would result in severe loss of fitness. Therefore, if a cancer cell harbours mutations in one of these genes, inactivation of the other would result in selective killing of the tumour cells. Recent advances in techniques may facilitate the identification of such gene-pairs in the near future (Kaelin 2006).

2.2.5 The hallmarks of cancer

Hanahan and Weinberg (2000) proposed six underlying principles that would define the nature and features of all cancers, namely self-sufficiency in growth signals, insensitivity to antigrowth signals, capability to evade apoptosis, limitless replicative potential, sustained angiogenesis, and capability to invade and metastasise, not necessary in this order. In contrast to the reductionistic approach only focusing on the cancer cells themselves, they emphasised the holistic view of cancer, according to which tumours are complex tissues comprising a number of different cell types, both

neoplastic and normal. Also, their model of tumorigenesis does not speculate on the number of independent (genetic) hits required as in some cases one hit can facilitate the acquisition of more than one of the six hallmarks or, on the other hand, in some cases multiple hits are required for the development of one hallmark.

2.2.6 Cancer stem cells

An important point, not discussed by the abovementioned models on cancer is the cell type from which the tumorigenic growth starts from. The idea that all cells are not prone to becoming tumours is not new, pointing to the direction of stem cells and, possibly, to cancer stem cells. Results from mostly haematological cancers suggest that only a small fraction of tumour cells are actually those maintaining the whole tumour cell population (Polyak and Hahn 2006). Several models on how a cancer stem cell developes have been proposed. A normal stem cell can acquire mutations, leading to the loss of asymmetric cell division and thus leading to increased amounts of stem cell-like progenitors, which then can acquire more mutations and become cancer stem cells. Alternatively, several mutations can occur in one stem cell, leading directly to the formation of a cancer stem cell that would supply the tumour with required non-stem cell-like cells. Still another possibility is that a fully or partially differentiated cell acquires mutations that make it transform into a stem cell-like state, for example through epidermal-mesenchymal transition. There is experimental evidence supporting all these pathways, and it may be, that in different tumour types, different pathways operate (Polyak and Hahn 2006). In accordance with the cancer stem cell model, a recent theory suggesting the epigenetic progenitor origin of human cancer has been proposed (Feinberg et al. 2006). There the authors suggest that the first step initiating the tumorigenic process is due to abnormalities in tumour-progenitor genes, which would result in vast epigenetic changes in a stem cell. These changes would then become present in a larger number of cells in the stem cell compartment. The second step, which is conceptually the same as the tumour initiation step in the Vogelstein model, would be a genetic alteration facilitating the tumour progression and subsequent invasion and metastasis. All tumour stem cell-based tumour models are supported by the notion that many of the hallmarks of cancer proposed by Hanahan and Weinberg (2000) are actually properties of normal stem cells. Therefore, no new genetic hits would be required for them to develop.

2.3 Tumour suppression: VHL as an example

As was the case with *p53*, it is not always clear whether a particular gene in which mutations occur in cancers acts as an oncogene or a tumour suppressor (Sherr 2004). However, as most of this work is motivated by the tumour suppressor genes, it is worth describing one of them in more detail. As in many cases, the *VHL* tumour suppressor gene was identified through the study of familial cancer. The first report describing the observation of bilateral vascular growths of the retinas in two siblings was published in 1894. Similar observations were soon made elsewhere and the hemangioblastomas of the central nervous system was also linked to the same syndrome. The term "von Hippel-Lindau (VHL) disease" was first used in 1936 and, since then, several different tumour types, such as clear-cell renal carcinomas, phaeochromocytomas, endolymphatic sac tumours of the inner ear, epididymal and broad ligament cystadenomas, and islet-cell tumours of the pancreas, have been linked to the syndrome. The prevalence of the VHL syndrome is about 1/35 000, and it is transmitted in an autosomal dominant manner (Kim and Kaelin 2004).

Genetic linkage analysis mapped the *VHL* gene to chromosome 3p25, a region that was known to be frequently deleted in sporadic renal cell cancers (Seizinger et al. 1988). This immediately suggested that the same gene might be involved both in sporadic and familial RCC. Five years later the gene was identified (Latif et al. 1993) and, at the same time, mutations were also detected in sporadic RCC. The gene was found to be evolutionarily conserved, but the predicted gene product did not show homology to any known proteins. Subsequent studies showed that *VHL* encoded a 30kD cytoplasmic protein, pVHL, and when reintroduced to a *VHL*-mutant RCC cell-line 786-O, it inhibited the ability of these cells to form tumours in nude mice (Iliopoulos et al. 1995). Thus, *VHL* was shown to act as a tumour suppressor gene, although the mechanism of action was still not known.

As many of the tumours associated with VHL were highly vascular, and as a rare paraneoplastic phenomenon, some patients affected with these tumours suffered from erythrocytosis due to overproduction of erythropoietin (EPO), a link between oxygen sensing and VHL-related tumours was hypothesised (Kim and Kaelin 2004). Further studies indeed corroborated this, as *VHL* negative cells were shown to over-express the vascular endothelial growth factor (VEGF), glucose transporter GLUT1, and platelet-derived growth factor (PDGF) mRNAs, even in normoxic conditions

(Iliopoulos et al. 1996). This function was dependent on pVHL forming a complex with Cul2, Elongin B, and Elongin C (Lonergan et al. 1998), which provided important clues into the function of pVHL, as the other members of the complex were homologous to yeast proteins involved in ubiquitin-mediated proteolysis. Thus, a role for VHL in ubiquitin-mediated degradation of proteins was proposed, which lead to the questions about the targets of this complex, and whether these targets could tell something about the link between VHL and hypoxia signalling.

Around the same time, another line of research had identified a novel transcription factor called hypoxia-inducible factor (HIF) through its activity to induce EPO expression (Semenza 1998). This factor had been shown to act as a heterodimer consisting of HIF1α and HIF1β subunits; HIF1β was stably expressed, but the HIF1α level was rapidly induced in hypoxia. Thus the question arose: Could HIF be the missing link between VHL and hypoxia? Evidence in favour of this idea soon came from the work of Maxwell et al. (1999) who showed that pVHL had a critical role in the oxygen-dependent degradation of HIF1α. The mechanism by which pVHL directs HIF1α for degradation was shown to be by recruiting the substrate to a ubiquitination machine (Ohh et al. 2000), which provided a mechanism for the pVHL-HIF1α interaction, although the basis for the oxygen-dependency of this reaction was not clear. Soon after though, two groups showed that an oxygen-dependent post-translational modification, namely the hydroxylation of specific prolyl residues of HIF1α was necessary for its degradation (Ivan et al. 2001, Jaakkola et al. 2001), and further studies on model organisms led to the identification of the HIF prolyl hydroxylase genes which in an oxygen-dependent manner hydroxylated these critical prolyls in HIF1α (Epstein et al. 2001). Although these studies provided mechanistic evidence linking VHL to hypoxia signalling, it was still not clear whether the same mechanism was responsible for tumour suppression.

Further evidence to support the role of pVHL in RCC formation came from a study that showed loss of *VHL* and induction of HIF-1 already in the early lesions of the kidney (Mandriota et al. 2002). By studying the tumour formation in nude mice, Kondo et al. (2002) reported that the ability of the 786-O cells to form tumours after reintroduction of VHL was rescued by expressing a HIF2α-mutant that was insensitive to prolyl hydroxylation, and thus to oxygen-dependent degradation. This suggested that HIF was indeed necessary for tumour formation. Furthermore, the same group showed that the down-regulation of HIF2α in VHL null cells was

sufficient to suppress tumour formation *in vivo* (Kondo et al. 2003). Together these studies showed that HIF2α was a critical down-stream factor of pVHL in renal tumorigenesis.

The VHL syndrome can be devided phenotypically into 2 different types according to the presence or absence of phaeochromocytoma (Maher and Eng 2002). Type 1 mutations, which normally result in total or partial VHL loss or improper folding, result in hemagioblastomas and RCC, but not phaeochromocytomas. Type 2a mutations result in hemangioblastomas and phaeochromocytomas with a low risk of RCC, while type 2b mutations have the same tumour spectrum as 2a but with an increased risk of RCC, and type 2c mutations predispose only to phaeochromocytomas. There is a genotype-phenotype correlation suggesting that complete loss of pVHL function would not result in phaeochormocytoma development. Moreover, genetic evidence shows that the *VHL* gene is lost either by somatic mutations or silencing by hypermethylation in about 30% of sporadic hemagioblastomas (Kim and Kaelin 2004) and in 50% of clear-cell RCCs (Gnarra et al. 1994, Herman et al. 1994, Kim and Kaelin 2004, Whaley et al. 1994), but truly somatic *VHL* mutations in phaeochromocytomas have not been detected (Neumann et al. 2002). Also, mutations in other tumour types are rare (Gnarra et al. 1994, Whaley et al. 1994). Furthermore, functional analyses of different *VHL* mutation types have suggested that type 2c mutations do not lead to over-expression of hypoxia inducible genes (Clifford et al. 2001, Hoffman et al. 2001). All this evidence supports the role of *VHL* as a classical tumour suppressor gene of the Knudson's two-hit hypothesis type in haemangioblastomas and RCC, but not in phaeochromocyotoma. This suggests that phaeochromocytoma development might require mutations in many cells (a field effect), or that the mutations would have to be present within a very specific time-window (Kim and Kaelin 2004). Evidence favouring the latter explanation has emerged recently, suggesting a role for VHL in neuronal apoptosis and developmental culling (Lee et al. 2005).

Together all these data on the tumour suppressor VHL highlight several characteristic features of the current tumour suppression paradigm (Sherr 2004). Firstly, inherited mutations in VHL predispose to a tumour syndrome which is transmitted in an autosomal dominant manner. Also, the sporadic counterparts of VHL associated tumours, at least RCC and haemangioblastoma, harbour mutations in the same gene. This is a great example of how the study of rare inherited syndromes

can tell a great deal about the genetic basis of common tumours. Secondly, both classes of tumours, sporadic and familial, show loss of the wild-type allele indicating that VHL acts recessively at the cellular level, which is a classical feature of a tumour suppressor gene. Thirdly, VHL seems to function as a gatekeeper for tumour initiation, which highlights its important role in early tumorigenesis. Fourthly, a genotype-phenotype correlation between different mutations and the clinical manifestations of the disease show that there is inherent logic in the pathobiology of these tumours. The functional studies have been able to dissect the rules behind these differences providing insight into the tissue specificity of tumours, a fundamental question of modern cancer research. Lastly, the discoveries on VHL function provides a beautiful example of how seemingly simple clinical observation can be combined with scientific outlook to bring new insight into our knowledge on common diseases, and how this knowledge can be further utilised for the benefit of patients in the form of more rationally developed targeted therapies.

2.4 Hereditary leiomyomatosis and renal cell cancer (HLRCC)

The syndrome hereditary leiomyomatosis and renal cell cancer (HLRCC) was first described in 2001 (Launonen et al.). The first hint of a new syndrome came from a patient that had been referred to genetic counselling due to apparent familial clustering of renal cell cancer in her family. Further studies of the medical records revealed that the RCCs were of a rare histological type, papillary type 2, and that uterine and cutaneous leiomyomas were also associated with the phenotype. In the course of the study, another family with the same type of RCC was identified, and subsequently a genetic linkage analysis mapped the disease locus to chromosome 1q42-q44. Several tumours showed loss of the unlinked chromosome, further supporting the involvement of 1q in the disease predisposition. In addition to leiomyomas and RCC, the affected females seemed to have an increased risk of leiomyosarcoma (Launonen et al. 2001). Soon after, another group published the localisation of a gene predisposing to multiple cutaneous and uterine leiomyomata (MCUL) to the same 1q42.3-q43 chromosomal region as HLRCC (Alam et al. 2001). These patients suffered from multiple cutaneous leiomyomas and uterine fibroids, but no predisposition to malignant tumours was observed in the 11 families studied. It was immediately suspected that HLRCC and MCUL were phenotypic variants of the

same syndrome, and evidence to support this was soon published as a family diagnosed with multiple cutaneous leiomyomatosis was found to be compatible with linkage to the 1q predisposition locus (Kiuru et al. 2001). Also, individuals in the family were found to be affected by RCC and leiomyosarcoma, further strengthening the link between MCUL and HLRCC. The cutaneous lesions were found to show LOH at 1q establishing the syndrome as a two-hit condition following Kundson's hypothesis (Knudson 1971). The MCUL and HLRCC teams joined for The Multiple Leiomyoma Consortium which in a short time identified the responsible gene, *fumarate hydratase* (*FH*) (Tomlinson et al. 2002). The consortium showed that in addition to having germline mutations, the FH enzyme activity was reduced in the lymphoblastoid cell lines from the carriers and the cutaneous tumours from these individuals showed very low or absent FH enzyme activity. Additional families with germline *FH* mutations displaying the HLRCC phenotype were later also published in North America (Toro et al. 2003). Screens for mutations and LOH in 1q showed that somatic inactivation of *FH* was rare in the sporadic counterparts of HLRCC associated tumours, although some 1q LOH was observed in uterine fibroids (Barker et al. 2002) and one soft tissue sarcoma with a somatic mutation and loss of the wild-type *FH* was found (Kiuru et al. 2002).

Fumarate hydratase (FH, fumarase) is an enzyme catalysing the hydration of fumarate to form malate in the Krebs tricarboxylic acid cycle (TCAC) in mitochondria (Stryer 1995). The functional enzyme is a homotetramer (Weaver et al. 1995) which is encoded by a nuclear gene (Tolley and Craig 1975). Both mitochondrial and cytosolic forms of FH exist (Edwards and Hopkinson 1979a, and 1979b). Homozygous mutations of *FH* result in the recessive disorder fumarase deficiency which manifests in severe developmental disorder with neurological and muscular involvement (Bourgeron et al. 1994, Gellera et al. 1990, Rustin et al. 1997). The involvement of a housekeeping gene like *FH* in tumour predisposition was a surprise, and several hypotheses for the tumorigenic mechanism were suggested, such as superoxide overproduction (Rustin 2002) or oxidative stress, pseudo-hypoxic drive, defect in apoptosis due to mitochondrial dysfunction, and anabolic drive (Eng et al. 2003, Pollard et al. 2003). Genotype-phenotype correlation for different manifestations of *FH* mutations regarding FH deficiency, MCUL and HLRCC could not be detected. Functional analysis showed loss or clear reduction of the enzyme

activity in all these syndromes therefore suggesting that all known *FH* mutations had very similar functional effects (Alam et al. 2003).

2.5 Other hereditary forms of RCC

Based on histological and genetic data, RCCs have been classified into distinct subtypes. These classes are: conventional (clear-cell); papillary, of which two distinct subtypes exist (types I and II); chromophobe; oncocytoma; collecting duct; and unclassified (Kovacs et al. 1997). The conventional and papillary RCCs are thought to arise from the epithelial cells of the proximal part of the renal tubule (Bodmer et al. 2002) whereas chromophobe, oncocytoma, and collecting duct tumours arise from the collecting duct cells (Bodmer et al. 2002, Cohen and McGovern 2005). As discussed above, the role of VHL in familial clear-cell RCC is well characterised (Bodmer et al. 2002, Cohen and McGovern 2005). In addition, constitutional translocations involving chromosome 3 have been shown to segregate with the conventional RCC phenotype, although the responsible genes have not as yet been identified (Bodmer et al. 2002).

Familial papillary RCC is caused by germline mutations in the *MET* proto-oncogene which is a transmembrane receptor tyrosine kinase that normally responds to the hepatocyte growth factor (Schmidt et al. 1997). These tumours are of papillary type I histology and seem to have a better prognosis than the type II tumours (Cohen and McGovern 2005). In contrary to classical tumour predisposition syndromes, the mutations in the *MET* kinase domain are of an activating nature, leading to ligand-independent growth promoting signalling (Jeffers et al. 1997). Instead of chromosomal deletions, amplification of the mutated allele occurs in tumours, multiplying the growth promoting signalling (Zhuang et al. 1998).

A rare autosomal dominant tumour predisposition syndrome, Birt-Hogg-Dubé syndrome (BHD), which is characterised by hair-follicle hamartomas of the face and neck, manifests in about 15% of cases also as renal tumours (Cohen and McGovern 2005). The renal lesions are typically chromophobe or mixed chromophobe-oncocytoma tumours, although papillary and clear-cell RCCs have also been detected in these patients (Schmidt 2004). The gene underlying the syndrome, *BHD*, has recently been identified (Nickerson et al. 2002) and it is suspected to act as a tumour

suppressor, as a second hit has been detected in several renal BHD tumours (Vocke et al. 2005). Mutations in sporadic tumours are detected only rarely (Schmidt 2004). The function of the *BHD* gene product, folliculin, is not known, although it is evolutionarily conserved (Nickerson et al. 2002). As both proximal and distal tubule tumours are associated with BHD, it has been suggested that folliculin regulates an early cell that still has the potency to differentiate into both proximal and distal tubule cells. Two animal models carrying germline mutations in the orthologous *BHD* genes have been described, both developing renal tumours (Lingaas et al. 2003, Okimoto et al. 2004).

Hyperparathyroidism-jaw tumour (HPT-JT) syndrome is an autosomal dominant multiple neoplasia condition characterised by hyperparatyreoidism caused by parathyroid tumours. Other manifestations are ossifying fibromas, most commonly affecting the mandible and maxilla (Jackson et al. 1990). Also, patients seem to have an increased risk of renal neoplasms, namely adult Wilms tumours, mixed epithelial-stromal tumours, multiple renal cysts, and sometimes also papillary renal cell carcinoma (Tan and Teh 2004). Interestingly, HPR-JT is associated with various uterine lesions such as adenosarcomas, adenofibromas, leiomyomas, adenomyosis, and endometrial hyperplasia (Bradley et al. 2005). Germline mutations in the *HRPT2* gene were recently identified in several HPR-JT families, and inactivating mutations were also detected in some sporadic parathyroid adenomas (Carpten et al. 2002). The inactivating nature of the mutations, together with results showing chromosomal somatic LOH at the *HRPT2* locus, indicate that the *HRPT2* product, parafibromin, might function as a tumour suppressor. Parfibromin is an evolutionarily conserved protein that is ubiquitously expressed (Carpten et al. 2002), and recent evidence shows that it may have a function regulating the Wnt-signalling pathway (Mosimann et al. 2006).

Tuberous sclerosis complex is an autosomal dominant syndrome predisposing to infantile seizures, mental retardation, autism, and tumours in the brain, retina, kidneys, heart, and skin (Astrinidis and Henske 2005). The renal manifestations are wide ranging including benign angiomyolipomas, which are composed of abnormal vessels, immature smooth muscle cells and fat cells, multiple renal cysts, oncocytomas, and renal cell carcinomas of clear-cell, papillary, and chromophobe histologies (Henske 2004). Tuberous sclerosis complex is caused by mutations in either *TSC1* (The European Chromosome 16 Tuberous Sclerosis Consortium 1993) or

TSC2 (van Slegtenhorst et al. 1997), which encode proteins hamartin and tuberin, respectively. They have been shown to interact and the complex has a well characterised function in modulating the mTOR signalling pathway (Wullschleger et al. 2006). That the renal tumours associated with TSC can be of both mesenchymal and epithelial origin has lead to the idea that tuberin and hamartin might be regulators of the early progenitor cells in the kidney (Henske 2004). The Eker rat, which harbours a germline mutation in the rat homologue of *TSC2*, has a phenotype of both renal cell carcinomas and uterine leiomyomas (Cook and Walker 2004).

2.6 Uterine leiomyomas

Uterine leiomyomas (also called fibroids or myomas) are benign tumours arising within the smooth muscle lining of the uterus, the myometrium. They are thought to originate from smooth muscle cells. Although benign, they cause significant morbidity in the form of abdominal pain, abnormal bleeding, and even infertility (Stewart 2001). Fibroids are the single most common cause for hysterectomies accounting for about 50% of these operations in Finland (Luoto et al. 1994). Therefore, fibroids represent a major burden on the health care system. Uterine fibroids are very common, affecting up to 77% of women (Cramer and Patel 1990), although clinically symptomatic fibroids are significantly less frequent (Stewart 2001).

Even though fibroids are very common, not much is known about their aetiology. Several risk factors have been detected in epidemiological studies. Early menarche, age, obesity, and the intake of red meat have been proposed as risk factors, whereas parity, smoking, and exercise have been shown to inversely correlate with the incidence of fibroids (Flake et al. 2003). A common denominator for all these risk factors might be oestrogen metabolism and, accordingly, oral contraceptives, hormone replacement therapy, and treatment with tamoxifen, a partial oestrogen agonist, have been shown to have effects on fibroids (Flake et al. 2003). However, different studies have contradicting results, and the exact role of oestrogens in fibroid development is not clear. Generally it is thought that these tumours are oestrogen dependent and that hormone replacement therapy, for example, would inhibit normal shrinkage of fibroids after menopause. Evidence supports this notion (Polatti et al. 2000). On the other hand, tamoxifen therapy seems to have growth promoting effects on fibroids

(Dilts et al. 1992), although *in vitro* studies have shown that it inhibits the growth of oestrogen stimulated growth of Eker rat derived uterine cell lines (Fuchs-Young et al. 1996).

Several lines of evidence suggest that genetic factors seem to have an impact on fibroid development. Firstly, from epidemiological studies there is evidence that the prevalence of fibroids is higher among black than white women (Flake et al. 2003). Also, studies on twins have shown a greater concordance for hysterectomy (Treloar et al. 1992) and hospitalisation due to uterine fibroids (Luoto et al. 2000) in monozygotic than dizygotic twins, and based on the recurrence rate in siblings, the heritability of fibroids has been estimated to be between 0.26 and 0.80 (Kurbanova et al. 1989, Luoto et al. 2000, Snieder et al. 1998). Although a rare example, HLRCC also represents evidence for the genetic predisposition to fibroids, and similar mechanisms could be involved in sporadic fibroid formation as well. Similarly, Alport syndrome, a rare condition which is sometimes associated with diffuse leiomyomatosis of the oesophagus, tracheobronchial, and genitourinary tract, is inherited in a dominant X-linked manner (Miner 1999). It is caused by germline deletions in collagen IV genes (Zhou et al. 1993), which could indicate a more widespread role of these extracellular proteins in common fibroids.

The clonality of uterine fibroids has been studied by methods that rely on the random inactivation of the X chromosomes during development. If a tumour is of clonal origin, the same X chromosome should be inactivated in all the cells, whereas if the origin of tumour is polyclonal, it should have developed from a heterogeneous group of cells in which some cells have inactivated one of the copies and others have inactivated the other copy (Fearon et al. 1987). The first report indicating that fibroids are clonal was published in 1965 (Linder and Gartler 1965) and several subsequent studies have confirmed these findings (Hashimoto et al. 1995, Mashal et al. 1994). In addition, as both karyotypically normal and abnormal cells have been shown to have similar X-chromosome inactivation pattern in individual tumours, it has been suggested that clonal expansion occurred before karyotypic changes (Mashal et al. 1994, Xing et al. 1997). Fibroids from the same patient have commonly different inactivated X-chromosomes suggesting independent development of multiple tumours instead of dissemination of only one tumour clone (Hashimoto et al. 1995, Linder and Gartler 1965, Mashal et al. 1994). Thus, the evidence shows that fibroids develop

through clonal expansion of one individual cell clone instead of a field effect, such as abnormal hormone secretion.

Traditionally, chromosomal changes in fibroids have been studied by cytogenetic methods. About 40-50% of fibroids show chromosomal (karyotypic, cytogenetic) changes, some of which are non-random (Sandberg 2005). The most common changes are t(12;14)(q15;q23~q24), del(7)(q22q23), rearrangements involving 6p21 and 10q, trisomy 12, and deletions of 3q. Fibroids with karyotypic abnormalities are larger than karyotypically normal fibroids, and also, fibroids carrying t(12;14) tend to be the larger and those carrying del(7) (Rein et al. 1998).

The most common change is t(12;14)(q15;q23~q24) occurring in about 20% of karyotypically abnormal fibroids. These regions are therefore thought to play a role in the pathogenesis of fibroids. The 12q15 region harbours a high mobility group protein encoding gene, *HMGA2*, which together with another protein from the same family, HMGA1, have higher expression in uterine fibroids compared to normal myometrium (Klotzbucher et al. 1999). Further studies on fibroids have identified aberrant *HMGA2* splice variants (Ingraham et al. 1999, Kurose et al. 2001, Schoenmakers et al. 1995) and fusion transcripts involving various other genes on different chromosomes such as *ALDH2* (Kazmierczak et al. 1995), *RAD51L1* (Schoenmakers et al. 1999, Takahashi et al. 2001), *COX6C* (Kurose et al. 2000), and *HEI10* (Mine et al. 2001). Later it has been postulated that the dysegulated HMGA2 expression, rather than aberrant fusion transcripts, would be of importance in fibroid development (Quade et al. 2003). *HMGA1*, on the other hand, is located at 6p21, a region which also shows recurrent changes in fibroids (Sandberg 2005). A study on the Eker rat showed HMGA2 expression also in murine tuberin-deficient fibroids. Taken together, these observations suggest that the HMG-proteins could have a role in leiomyomagenesis, although direct functional evidence of this is still lacking.

Recurrent interstitial deletions of chromosome 7q ranging from q11.23 to q36 were originally detected in cytogenetic studies (Sandberg 2005), and subsequently a number of molecular allelotyping studies have further characterised these changes (Ishwad et al. 1995, Ishwad et al. 1997, Mao et al. 1999, Sell et al. 1998, van der Heijden et al. 1998). The studies have pointed to a commonly deleted region within the chromosome region 7q22-q31 (Figure 2), but although several genes from this region have been proposed as having a role in leiomyomagenesis, no robust genetic evidence for this has been produced. Despite some mutation screening efforts (Barker

et al. 2002, Patrikis et al. 2003), the only point mutations in fibroids reported before this study, in addition to those associated with HLRCC, were 3 cases of *KRAS* mutations (Hall et al. 1997).

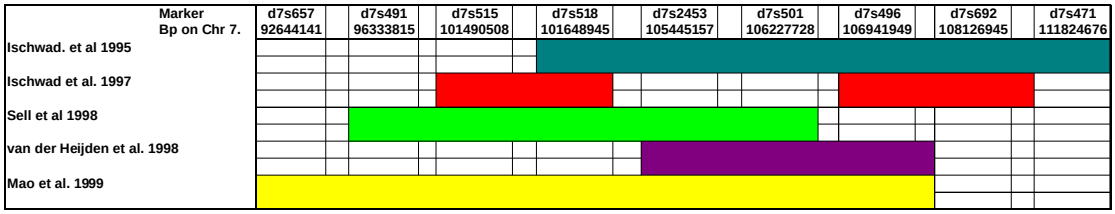

Figure 2. Minimal chromosome 7q21.3-q31.1 deleted regions detected in previous allelotyping studies on uterine fibroids.

The global gene expression pattern in uterine fibroids has been studied previously on several different platforms (Ahn et al. 2003, Arslan et al. 2005, Catherino et al. 2003, Chegini et al. 2003, Hoffman et al. 2004, Quade et al. 2004, Skubitz and Skubitz 2003, Tsibris et al. 2002, Wang et al. 2003, Weston et al. 2003), and great expectations have been laid on the genome-wide approach on uterine fibroid biology (Freije 2003). Some commonly deregulated genes have been identified, although the results from different experiments are largely non-overlapping (Arslan et al. 2005). The variation is likely due to the wide range of expression microarray and data analysis methods utilised in these studies (Hoffman et al. 2004, Tsibris et al. 2003). The small number of samples has compromised the statistical power of several of these studies and, also, none of them have taken into account different genetic statuses of the tumours.

2.7 Familial paraganglioma

Paragangliomas are tumours of the neurogenic origin, and they arise within the paraganglia that are dispersed throughout the body (Baysal 2002). The paragangliomas in the head and neck (HNP) regions are associated with the parasympathetic nervous system and are normally hormonally silent. The most common site of HNPs is the carotid body, although they are more than 20 different locations where they can occur. Paragangliomas arising below the neck, phaeochromocytomas, on the other hand, are more closely associated with the sympathetic nervous system, and they often give symptoms, such as hypertensive

crises, due to catecholamine secretion. The most common location of phaeochromocytomas is within the adrenal gland (Baysal 2002).

The first locus (*PGL1*) for hereditary paragangliomas (HPGL) was mapped by classical linkage analysis to chromosome 11q23-qter, and the pattern of inheritance suggested genomic imprinting as affected females did not transmit the disease (Heutink et al. 1992). Subsequent studies showed that LOH is not common in paragangliomas, apart from chromosome 11 which is lost in the chief cell compartment of these tumours (Devilee et al. 1994, van Schothorst et al. 1998). This suggested that PGL1 was a tumour suppressor gene, and that chief cells formed the neoplastic component of the tumours. After extensive fine mapping efforts (Baysal et al. 1999, van Schothorst et al. 1996), the PGL1 gene was identified later as *SDHD* and it was the first nuclear encoded mitochondrial gene to be linked to neoplasia (Baysal et al. 2000). The mutations were predicted to be truncating in most of the cases. A strong founder effect has also been detected in Dutch PGL1 families, as most patients have been shown to be affected by only a few mutations (Taschner et al. 2001, van Schothorst et al. 1998). As phaeochromocytomas and paragangliomas were known to be related, it was no great surprise when *SDHD* mutations were detected also in patients and families with phaeochromocytoma (Gimm et al. 2000).

Subsequent studies investigated the role of the other genes encoding SDH in patients with paraganglioma or phaeochromocytoma. Mutations in *SDHC* (*PGL3*) were mapped (Niemann et al. 2001) to chromosome 1q21-q23 and identified (Niemann and Muller 2000) in a single German family, and *SDHB* (*PGL4*) mutations on chromosome 1p36 were detected by candidate gene screening (Astuti et al. 2001). One additional locus, PGL2, has been mapped to chromosome 11q12-q14 (Mariman et al. 1993), but the responsible gene has not been identified. In contrast to *SDHB*, *SDHC*, and *SDHD*, no mutations in the fourth SDH gene, *SDHA*, have been detected in tumours, but homozygous mutations in *SDHA* result in Leigh syndrome (Bourgeron et al. 1995), a severe developmental disorder similar to FH deficiency. There are several possible explanations as to why *SDHA* mutations do not predispose to cancer. The discovery of a second gene encoding *SDHA* (Tomitsuka et al. 2003), if proven to be functional, would make it plausible to speculate that in order to develop full SDHA deficiency, 4 hits would be required, which is highly unlikely (Gottlieb and Tomlinson 2005).

Most HPGL tumours seem to have a second hit, suggesting that the SDH genes are tumour suppressors (Gottlieb and Tomlinson 2005). In the case of *SDHB* and *SDHC*, the wild-type allele is normally lost by a deletion. *SDHD*, on the other hand, was first thought to be imprinted (Heutink et al. 1992) but further studies have shown biallelic expression of *SDHD* in several tissues (Baysal et al. 2000). It has therefore been proposed that the imprinted gene/genes are only linked to *SDHD*, which is supported by the observations that the whole maternal chromosome 11 seems to be lost in PGL1 tumours (Hensen et al. 2004). Thus, the chromosomal deletion of the maternal wild-type *SDHD* would also result in the loss of another, maternally expressed tumour suppressor.

The SDH complex is formed by 4 different subunits. SDHC and SDHD are anchor proteins that connect the holoenzyme to the mitochondrial membrane. SDHA, the flavoprotein, and SDHB, the iron-sulphur protein, form the catalytic domain (Yankovskaya et al. 2003). SDH takes part in two different metabolic pathways, the Krebs cycle and the electron transport chain, in which it forms the mitochondrial complex II. There are 5 mitochondrial complexes; complexes I to IV function as electron transporters and complex V as a ATP synthase (Eng et al. 2003). No mutations in tumours have been detected in the genes encoding the mitochondrial complexes other than complex II, but defects in these complexes can result in developmental disorders (Eng et al. 2003). Therefore, SDH defects could be predicted to result in a different phenotype than mutations in *FH*, which does not function in the electron transport.

Carriers of *SDHB* mutations seem to have an increased risk of adrenal phaeochromocytoma and lower risk of paraganglioma compared with patients having mutations in *SDHD* (Neumann et al. 2004, Pawlu et al. 2005). Mutations in *SDHD* seem to increase the risk of multifocal tumours, and *SDHB* mutations that of malignant extra-adrenal paragangliomas (Gimenez-Roqueplo et al. 2003). *SDHB* mutations are more likely to abrogate SDH function in the TCA cycle than mutations in *SDHD*, which could explain some of the phenotypic differences. However, if all SDH mutations result in complete loss of SDH function, as has been suggested, these differences are hard to explain (Gottlieb and Tomlinson 2005). *SDHC* mutations are rarer and they seem to cause a benign phenotype (Schiavi et al. 2005).

Other syndromes that include predisposition to phaeochromocytomas or paragangliomas are VHL (discussed above), multiple endocrine neoplasia type 2

(MEN2), and neurofibromatosis 1 (NF1) (Maher and Eng 2002). MEN2 is caused by germline mutations in the *RET* proto-oncogene, and it can be divided into 3 clinically distinct subtypes: MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC). MEN2A, which is the most common subtype, is characterised by medullary thyroid carcinoma, phaeochromocytoma and hyperparathyroidism. MEN2B has the same tumour spectrum as MEN2A, but the symptoms start about 10 years earlier. The last form, FMTC, is characterised by MTC only. *RET* mutations have been detected in about 95% of MEN2 cases. There is a genotype-phenotype correlation between the 3 different forms of MEN2 and the position of the mutation, although some overlap has been detected. Altogether, about 50% of MEN2 patients develop phaeochromocytoma. NF1 is caused by germline mutations in the *NF1* tumour suppressor, and the risk of phaeochromocytoma in these patients is between 0.1% and 5.7% (Opocher et al. 2005).

2.8 From mitochondrial dysfunction to neoplasia

Mutations in genes encoding two nuclear encoded mitochondrial enzymes, FH and SDH, have been implicated in cancer. SDH functions in the TCA cycle and converts succinate into fumarate, and FH continues from there, converting fumarate into malate. Therefore, the TCA cycle appears as a natural denominator for both FH- and SDH-associated neoplasia syndromes. However, SDH also has a role in the mitochondrial electron transport chain. As described above, the tumour spectra of these mutations do not overlap, which clearly indicates that the tumorigenic mechanisms cannot be identical. Still, several hypotheses have been proposed that try to link both the mitochondrial dysfunction and the biology of these two syndromes to tumorigenesis. These theories include a decrease in apoptosis, an increase in the production of reactive oxygen species (ROS), and the activation of hypoxia signalling under normoxic conditions (pseudo-hypoxia) (Eng et al. 2003, Gottlieb and Tomlinson 2005, Pollard et al. 2003).

It is well established that mitochondria have an essential role in apoptosis (Hengartner 2000), and that mitochondrial metabolims and apoptotic mechanisms are linked (Downward 2003). Therefore, it seems plausible that defects in mitochondrial metabolic enzymes could lead to defects in apoptosis. Indeed, there is some evidence that mutations in *SDHC* could inhibit cellular responses to various apoptotic signals

(Albayrak et al. 2003). Also, downregulating SDHD has been shown to protect cells from apoptosis (Lee et al. 2005). Still it is not clear whether the blockage of the TCA cycle and/or the electron transport chain would always lead to a decrease in apoptosis, and alternative hypotheses have to be kept in mind (Gottlieb and Tomlinson 2005).

Increased ROS production has also been detected in SDH-deficient cells (Albayrak et al. 2003, Ishii et al. 2005). This is mainly thought to result from the disruption of the electron transport chain, and not from the blockage of TCA cycle and, therefore, *FH* mutations are not predicted to result in increased amounts of ROS. It has been postulated that a mild increase in ROS could be beneficial for tumours (Rustin 2002). Oxidative damage could also cause mutations in nuclear DNA, some of which could be important for tumorigenesis (Gottlieb and Tomlinson 2005). Another link from ROS to tumorigenesis could be through the induction of HIF signalling. There is evidence that ROS can activate HIF by inhibiting HIF prolyl hydroxylases (Gerald et al. 2004). The activation of HIF, on the other hand, can have several beneficial effects on a tumour cell, such as increased aerobic glycolysis (Gatenby and Gillies 2004). The activation of HIF in SDH- and FH-deficient cells can also be achieved through a mechanism independent of ROS. Recent results have shown that blocking FH or SDH activity results in the accumulation of fumarate and succinate, respectively, and they both can inhibit HIF prolyl hydroxylation (Isaacs et al. 2005, Selak et al. 2005). In this model, succinate and fumarate act as internal signalling molecules that transmit the message from mitochondria to cytosol (Gottlieb and Tomlinson 2005).

3 HYPOTHESES

The following 4 hypotheses form the basis of this work:

H1) A subset of early-onset renal cell carcinomas (RCC) is caused by HLRCC

H2) Chromosome 7q21-31 harbours a leiomyoma suppressor gene

H3) Uterine leiomyomas carrying a 7q-deletion form a biologically distinct subgroup of leiomyomas

H4) Fumarate hydratase-deficient uterine leiomyomas form a biologically distinct subgroup of leiomyomas

4 METHODOLOGY

4.1 LOH mapping and genomic sequencing (I, II, III, IV)

Both loss of heterozygosity (LOH) mapping and genomic sequencing rely fundamentally on the polymerase chain reaction (PCR), which enables exponential amplification of the target sequence. This cyclic method of enzymatic amplification of DNA *in vitro* was originally introduced in 1985 (Saiki et al. 1985). In the beginning, thermolabile Klenow fragment of *Escherichia coli* DNA polymerase I was used to catalyse the extension reaction of the primers, but only the introduction of the DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (*Taq*) facilitated wider use of PCR (Saiki et al. 1988). This development greatly simplified the procedure as no longer was it necessary to add more enzyme after each denaturation step. Also, due to higher extension temperatures, the reaction became more specific and efficient, making sequencing of various and even closely homologous target sequences possible (Saiki et al. 1988).

LOH or, more precisely, allelic imbalance (AI), is measured by comparing the amount of PCR products amplified from the two constitutional chromosomes. This is possible when the sequence amplified contains a polymorphism that distinguishes between the two alleles. Typically, the polymorphic sequences that are utilised for LOH mapping are microsatellite repeats although, for example, single nucleotide polymorphisms can be used for this as well. Microsatellites are short tandem repeats of very simple DNA sequences, usually 1-4 bp long, for example $(GT)_n$, that are widely dispersed throughout the genome. Probably because of their repetitive structure, they are prone to replication errors and unequal crossover, and therefore they are usually highly polymorphic (Strachan and Read 1999). Mononucleotide repeats are usually very polymorphic, but they are also prone to replication errors during the PCR. Therefore, di- tri- and tetranucleotide repeats are commonly used when assessing LOH.

We wanted to analyse uterine leiomyomas for LOH at two regions of interest, chromosome 1q43, the region where *FH* is located, and 7q21-q31, which is commonly deleted in uterine leiomyomas. For 7q we identified 25 microsatellite markers covering the whole region that according to previous reports harbours the minimal deleted region. For 1q, only two markers flanking the *FH* locus were utilised,

as the assumed target, the *FH* gene, was already known. The markers were chosen utilising the Ensembl genome browser (www.ensembl.org) (Birney et al. 2006) that contains marker information from various genetic maps, such as Marshfield (Broman et al. 1998) and Genethon (Gyapay et al. 1994). When no previously described markers where available, the genomic target region was searched for microsatellite repeats by Tandem repeats finder (Benson 1999) and PCR primers were designed by the Primer3 program (Rozen and Skaletsky 2000). Fluorescence-labelled primers (Sigma-Genosys, Cambridgehire, UK) were used to amplify matching normal and tumour DNA with PCR. The PCR products were detected with the ABI377 sequencer and analysed with Genotyper 2.5.2 software (Applied Biosystems, Foster City, CA). LOH was scored by calculating the intensity ratio of the constitutional alleles as described before (Canzian et al. 1996). Intensity ratio $L < 0.60$ or $L > 1.67$ was called LOH, $0.60 < L < 0.75$ or $1.33 < L < 1.67$ was called marginal LOH and $0.75 \leq L \leq 1.33$ was called normal heterozygosity.

For genomic sequencing we utilised a method based on the combination of Sanger sequencing (Sanger et al. 1977) and fluorescence detection (Prober et al. 1987, Smith et al. 1986). Over the years, this approach has become more efficient through 'evolutionary' advancements (Metzker 2005), and its power has been proved by the success of the Human Genome Project (Collins et al. 2003, International Human Genome Sequencing Consortium 2004, Lander et al. 2001).

For the leiomyoma candidate gene screening on 7q, gene information was obtained from Ensembl (Birney et al. 2006) and UCSC (Kent et al. 2002) genome browsers. Genomic PCR primers for all known manually annotated protein coding genes (Ashurst et al. 2005) within the minimal deleted region were designed utilising the Primer3 program (Rozen and Skaletsky 2000). The primers were designed to amplify coding exons and the flanking splice site junctions. The PCRs were carried out in a 25 or 50 µl reaction volume containing 50 or 100 ng of genomic DNA, respectively, 1x PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 300 µM each deoxynucleotide triphosphate (dNTP) (Finnzymes, Espoo, Finland), 1 µM forward and reverse primer (Sigma-Genosys, Cambridgehire, UK), 1.25 or 2.5 units of AmpliTaq Gold polymerase, (Applied Biosystems), respectively, and 1.5 to 3 mM $MgCl_2$. The general cycling conditions were as follows: 10 min at 95°C; followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55 to 62°C for 1 min, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min. A 5 µl aliquot

of the PCR product was run on an agarose gel to verify the specificity of the reaction, and the rest of the product was purified using the NucleoSpin PCR purification kit (Macherey-Nagel, Düren, Germany) or by ExoSAP-IT (USB, Cleveland, Ohio, USA). Direct sequencing of PCR products was performed using Applied Biosystems 3100 and 3730 BD3.1 sequencing chemistry and 5.1 sequencing analysis software (Applied Biosystems). The mutation screening of *FH* and all 4 genes coding the subunits of SDH, *SDHA*, *SDHB*, *SDHC*, and *SDHD* was essentially performed as outlined above. The primer sequences for each gene were obtained from literature (Astuti et al. 2001, Baysal et al. 2000, Kiuru et al. 2002), except for *SDHA*, for which primers were designed.

4.2 Microarray comparative genomic hybridisation (II, IV)

In addition to LOH mapping, microarray comparative genomic hybridisation (aCGH) was performed to further characterise the changes observed in uterine leiomyomas. The aCGH method is based on simultaneous hybridisation of differentially labelled tumour and normal DNA on microarray chips that contain short fragments of DNA as probes. We performed two different aCGH experiments. The first array contained 3 452 large genomic bacterial artificial chromosome (BAC) DNA clones at an average spacing of about 1 Mb throughout the genome. The second array was produced from two separate sets of BAC DNA clones; the first set consisted of 573 clones selected from the array of 3452 clones used in the 1 Mb resolution array, and a second set consisted of 1083 clones from chromosome 7q at a median midpoint spacing of 90 kb, obtained as a part of the "32k" BAC array produced by Osoegawa et al. (2001). Non-specific hybridisation was controlled by 8 random drosophila clones that have been shown not to hybridise to human chromosomes by fluorescence *in situ* hybridisation.

The microarrays were constructed by amplifying the clones by DOP-PCR using three different primers, followed by pooling and amplification by amino-linked PCR primer and printing on Codelink coated slides (Amersham, Uppsala, Sweden), as previously described (Douglas et al. 2004, Fiegler et al. 2003). The tumour DNA from uterine leiomyomas and reference DNA from pooled normal tissue samples were labelled with Cy5- and Cy3-dCTP, respectively. Both, the leiomyoma and normal reference samples were co-hybridised to the arrays with herring sperm DNA and COT human DNA (Roche, Lewes, United Kingdom), followed by washing and scanning

with a confocal scanner (Perkin Elmer, Wellesley, MA, USA). Samples with poor hybridisation were rejected by visual inspection after which the Spot program (Jain et al. 2002) was used for the image data quantification. After correcting for background based on the drosophila clones, $\log_2$ ratio of the fluorescence intensities of tumour to reference was calculated. Standard deviations of these ratios were calculated genome-wide. Tumour-normal ratios of greater than 3 were taken to indicate gain and ratios of less than -3 were scored as loss.

4.3 Expression microarray analysis (II, III)

Global gene expression patterns in uterine fibroids and corresponding normal myometrium was measured by oligonucleotide microarray chips, a method proven extremely powerful in many recent studies on various tumours (Golub et al. 1999, Singh et al. 2002). The technology is based on a combination of solid-phase chemistry, photolabile protecting groups and photolithography, which facilitates the parallel synthesis of various chemical compounds (Fodor et al. 1991). This approach makes it possible to produce high density arrays of various biological compounds *in situ* based on sequence information alone (Fodor et al. 1993). The expression of tens of thousands of genes can therefore be measured in a single experiment (Lockhart et al. 1996, Wodicka et al. 1997).

4.3.1 Experimental procedures

For the expression microarray experiments, double-stranded cDNA was produced from 8 micrograms of total RNA by using the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, CA). The cDNA were purified using phenol-chloroform-isoamyl alchohol solution (Amresco, Solon, Ohio) and Phase Lock Gel™ Heavy 1.5 ml tubes (Eppendorf AG, Hamburg, Germany), followed by ethanol precipitation. For the production of biotin labelled cRNA, *in vitro* transcription was performed by RNA Transcript Labeling Kit (Enzo Diagnostics Inc, Farmingdale, NY). The labelled cRNA was then purified with RNeasy® mini columns (Qiagen Ltd) and fragmented by metal ion catalysed cleavage at 94°C for 35 minutes in the presence of 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate. Fifteen micrograms of fragmented cRNA with 0.1 mg/mL herring sperm DNA in MES buffer were hybridised on GeneChip® HG-U133A oligonucleotide chips (Affymetrix Inc, Santa Clara, CA) at 45°C for 16 hours.

Subsequently, the chips were washed and stained with streptavidin-phycoerythrin using Fluidics Station 450 (Affymetrix) and scanned using GeneChip® System GA2500 Scanner (Affymetrix). All procedures were carried out as outlined in the Affymetrix technical manual (Affymetrix Inc. 2004).

4.3.2 Expression data analysis

Quantitative information was derived from scanned images using MAS 5.0 software (Affymetrix). Subsequently, the dChip 1.3 PM/MM difference model was used to check for single, probe and array outliers, in addition to the preliminary hierarchical clustering (Li and Wong 2001). The expression values of all genes called marginal or present at least in 50% of the samples were selected and the data were $\log_2$ transformed and arrays were centred to the median.

For unsupervised clustering (Eisen et al. 1998), gene vectors with small standard deviation were filtered out, typically resulting in a list of about 1000 genes that were the most differentially expressed across the samples. Subsequently, the arrays were organised with self organising maps, and hierarchically clustered using Pearson correlation coefficient as the similarity metric. The cluster tree images were created with TreeView 1.60 software (Eisen et al. 1998). Another unsupervised classification method, principal component analysis (PCA), was also performed to evaluate the global differences between different groups of fibroids. This was done by utilising the TIGR MeV software (Saeed et al. 2003) and PCA method as described (Raychaudhuri et al. 2000).

Differentially expressed genes were detected using Significance Analysis of Microarrays (SAM) software (Tusher et al. 2001). In all SAM analyses, missing values were imputed with K-Nearest Neighbors Imputer (10 neighbours) and a two-class test for unpaired data was used with 5000 permutations. In addition to only looking at individual deregulated genes, a functional group enrichment analysis (FGEA) was performed to analyse the expression data in biologically meaningful subgroups. For FGEA, we utilised GoMiner (Zeeberg et al. 2003) software firstly, to find Gene Ontology (GO) (Ashburner et al. 2000) annotations for all the genes in the microarray analysis and, secondly, to perform a two-sided Fisher's exact test to calculate p-values for the enrichment of each GO category in the list of differentially expressed genes.

In order to find out whether the expression levels of a small set of genes could be used to classify fibroids as being either *FH*-mutant or *FH* wild-type, a molecular classifier was developed with the method described by Golub et al. (1999). Firstly, the Finnish set of samples was used in a leave-one-out cross-validation procedure[1] for testing the performance of predictors consisting of 1 to 10 different genes. The results from this test guided the selection of the number of genes chosen for the final predictor which was further validated on the independent set of UK fibroids. The class assigned for tested samples was determined by 3 of its nearest neighbours using the Cosine distance metric. The molecular predictor analysis was performed utilising Gene Cluster software (Reich et al. 2004).

4.4 Quantitative real-time RT-PCR (II, III)

To confirm some of the findings discovered by expression microarray analyses, we performed additional experiments by quantitative real-time reverse transcriptase (RT) PCR. Firstly, complementary DNA (cDNA) is produced from total RNA extracted from the samples. Then, a PCR is performed during which the accumulation of the PCR product is assessed in real time by detecting the fluorescence signal produced by the product specific probe. As *Taq* DNA polymerase has 5' à 3' exonuclease activity, it cleaves the probe during the PCR, if the probe hybridises with the target sequence (Holland et al. 1991). The probe contains a reporter fluorescence dye, which is quenched by another dye at the other end of the probe. This cleavage separates the reporter dye from the quencher and therefore increases the reporter dye fluorescence signal (Lee et al. 1993). The progress of the PCR can be monitored in real time as the accumulating intensity of the reporter dye (Mullah et al. 1998).

We measured the relative expression of *DAPK1*, *RARRES3*, *RINT-1*, *TKT*, *LDHA*, *CDKN1A* and *AATF* in fibroid and normal myometrium samples. *TATA-box binding protein* (*TBP*) and *β-actin* (*ACTB*) were utilised as endogenous control genes, as their expression did not vary significantly as measured by expression microarray analysis. Firstly, cDNA were synthesised from total RNA utilising M-MLV reverse

[1] The leave-one-out cross-validation goes briefly as follows (Golub et al. 1999): one sample is withheld and the remaining samples are sorted according to the correlation with the phenotype; the genes with the best positive or negative correlation are chosen for a predictor, which is then utilised for determining the phenotype of the withheld sample. The procedure is repeated until all samples in the initial dataset have been tested. The accuracy of the predictor is calculated as the proportion of erroneous predictions. The final predictor is built based on all samples in the initial data set. This predictor can be tested in an independent set of samples.

transcriptase (Promega, Madison, WI) and 300 ng of random hexamer primers in the manufacturer's buffer containing 1 mmol/l of dNTP, and 40 U of RNase inhibitor (Promega). The reverse-transcriptase reaction was as follows: 42°C for 50 min, followed by 95°C for 10 min and 4°C for 5 min. Subsequently, the cDNA was amplified by PCR, and the accumulation of the product was detected in real time utilising an ABI PRISM SDS5700 sequencing detector system (Applied Biosystems) and TaqMan® Gene Expression Assays (Applied Biosystems). The RT-PCR reactions were done in triplicate. The relative expression of each gene was calculated by the equation 2^{-ddCt}, where Ct is the mean threshold cycle of the PCR reaction at which the accumulation of the PCR product is exponential, dCt is the difference between the threshold cycle of the target and control genes, ddCt is the normalised dCt value derived from subtracting the highest dCt from all other dCt values of the respective gene in each experiment. The statistical significance was tested by a two-sided heteroscedastic t-test in the cases where the distribution of the data was close to normal; The Mann-Whitney U test was performed when the data distribution was skewed.

4.5 Western blot analysis (III)

For measuring FH protein expression in uterine fibroids, we used a method called Western blotting that was first described in 1981 by Burnette (1981). It is based on polyacryl amide gel electrophoresis that separates the denatured proteins according to their size, the blotting of the protein out of the gel and onto a nitrocellulose or polyvinylidene fluoride (PFVD) membrane, and subsequent detection by protein specific primary antibody and primary antibody specific secondary antibody.

Total protein was extracted from fresh frozen uterine fibroid specimens using the T-PER™ Tissue Protein Extraction Reagent (Pierce, Rockford, IL) protocol and the protein concentration was determined by BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). After denaturation, 5 µg of protein was separated on a 10% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and blotted onto a PVDF membrane (Millipore, Bedford, MA). The membranes were probed with purified polyclonal rabbit anti-porcine FH antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) at a 1:500 dilution. After stripping of the FH antibody, the same membrane was probed with monoclonal mouse α-tubulin antibody at a 1:10 000

dilution (Sigma-Aldrich, St. Louis, MO) to obtain a protein loading control. Both FH and α-tubulin were detected by the ECL plus Western Blotting Detection System (Amersham Biosciences UK Ltd, Buckinghamshire, England).

5 TEST MATERIALS

All patient materials were collected either anonymously or with the informed consent by the patient, and the collection was approved by the local ethics committee.

5.1 Renal cell carcinomas (I)

The Finnish Cancer Registry – Institute for Statistical and Epidemiological Cancer Research was established in 1953, and since then it has kept a record of all cancer cases in Finland by receiving information from hospitals, practicing physicians and pathological and haematological laboratories. Also, death certificates in which cancer is mentioned are transferred into the registry each year (Finnish Cancer Registry – Institute for Statistical and Epidemiological Cancer Research 2005). As reporting to the registry is mandatory by law, and as the mortality follow-up is extremely efficient in Finland due to the use of personal identification numbers since 1967, the coverage of the national cancer registry is almost complete (Teppo et al. 1994). The data are also of high quality as less than 1% of the cases have death certificates as the only source of clinical information (Dickman et al. 1999), and as the histological confirmation of cancer is received in more than 90% of cases (Finnish Cancer Registry – Institute for Statistical and Epidemiological Cancer Research 2005). We searched the registry for early-onset renal tumours of all histological types diagnosed from ages 15 to 34, after which the pedigrees were extended and documented utilising data from the Finnish population registry and patient records. Younger patients were left out due to the high proportion of Wilms tumours in that age group (Haber 2002). A set of 60 sporadic RCCs that comprised 30 clear-cell, 3 papillary, 2 granular cell, 1 mixed papillary/clear-cell, 1 mixed clear-cell/solid histology, 9 oncocytic papillary, and 14 oncocytoma cases diagnosed at any age were collected at Helsinki University Central Hospital and at Jyväskylä Central Hospital.

5.2 Uterine leiomyomas (II, III, IV)

A set of 166 sporadic uterine leiomyomas were collected from 51 patients undergoing a hysterectomy at Helsinki University Central Hospital. The tumour and corresponding normal myometrium samples were snap-frozen in liquid nitrogen and subjected for further analysis anonymously. In addition, 6 uterine leiomyomas and corresponding myometrium were obtained from a patient carrying a germline *FH* mutation 541delAG (Tomlinson et al. 2002). The DNA was extracted by the Fast DNA® Kit (Bio 101, Carlsbad, CA, USA), which utilises ceramic spheres and a rapid shaking and twisting motion at very high speeds to homogenise the tissue sample rapidly, or by a standard non-enzymatic method (Lahiri and Nurnberger 1991). Of the samples subjected to expression microarray studies, frozen sections were haematoxylin-eosin-stained and the diagnosis was confirmed. The mitotic rate was assessed and cellularity was semi-quantitatively graded from 1 to 3, with 1 defined as high, 2 as medium and 3 as low stroma/nuclei ratio. RNA was extracted using the Trizol® reagent (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) and an RNA clean-up with RNeasy® mini columns (Qiagen Ltd, West Sussex, UK) was performed. For the expression microarray experiment, RNA integrity was verified by an Agilent 2100 Bioanalyzer RNA 6000 Nano Chip (Agilent Tecnologies, Waldbronn, Germany) analysis. The samples with poor RNA quality were subjected to a second round of RNA extraction. A separate sample set for expression microarray studies was obtained in The United Kingdom: 3 samples of normal myometrium; 2 sporadic, *FH* wild-type fibroids; and 5 *FH*-mutant fibroids from a HLRCC patient carrying a germline *FH* mutation K187R (Tomlinson et al. 2002). Also, DNA from a UK set of 174 sporadic uterine fibroids was utilised for LOH and aCGH analyses.

6 RESULTS

6.1 Renal cell canrcinomas (I)

The computerised search of the Finnish Cancer Registry revealed 244 unrelated patients with RCC diagnosed at age 15 to 34. Cases with diagnosed VHL and Wilms tumour were excluded, in addition to cases diagnosed before the year 1985, as older paraffin embedded tissue blocks would have been hard to obtain. This left 84 index cases, for which the pedigrees were expanded to include first degree relatives and their cancer status derived from the cancer registry records; 19 interesting families suggesting possible inherited cancer were found, including Family A (Figure 3). For the remaining 18 families, the investigations are still ongoing. The index case had received the diagnosis of RCC at age 28, when the tumour had already metastasised. The kidney was resected in a palliative operation. Histologically, the tumour was defined as a clear-cell carcinoma that showed a mixture of clear cells and cells with granular-eosinophilic cytoplasm. In the cancer registry records, the proband's mother was defined as having suffered from a malignant tumour of the heart, which in further examination of the patient records turned out to be a malignant paraganglioma growing from the septum to the right ventricle. She received the diagnosis at the age of 55, and as the tumour was considered inoperable, it subsequently led to the demise of the patient. Two of the proband's maternal uncles had also suffered from cancer; one had a small-cell lung carcinoma diagnosed at age 55, and the other pancreatic carcinoma diagnosed at age 71.

To test the hypothesis that early-onset renal cell cancer is caused by the syndrome HLRCC, DNA from these samples was derived from archived paraffin embedded tissue blocks and subjected to *FH* mutation analysis. No changes were observed, however. As mutations in the genes encoding subunits of SDH had been detected in familial paragangliomas, an alternative hypothesis of SDH involvement in the tumour predisposition in Family A was tested by directly sequencing all 4 SDH genes, *SDHA*, *SDHB*, *SDHC*, and *SDHD*. This revealed a truncating mutation R27X in the *SDHB* gene. Both the proband and his mother carried the mutant allele, and the wild-type allele was lost in the proband's RCC and in the mother's cardiac PGL. The maternal uncle with lung cancer was also a carrier of the mutant allele, but no loss of the wild-type was observed. From the uncle with the pancreatic cancer, no DNA was

available for analysis. In the further screen of all four SDH genes in the set of 60 mixed sporadic and 35 clear-cell RCCs diagnosed before the age of 50, no mutations were detected.

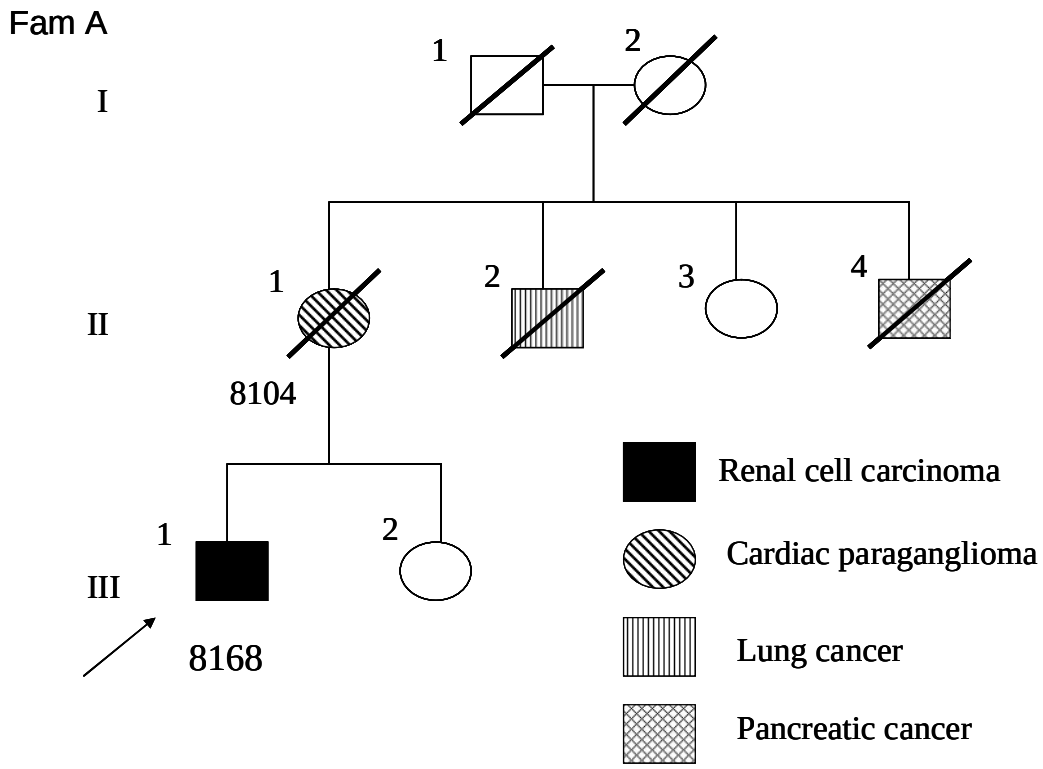

Figure 3. Family A ascertained through the search at the Finnish Cancer Registry database.

6.2 Uterine leiomyomas (II, III, IV)

6.2.1 Leiomyoma histology (II, III)

To confirm and scrutiny the diagnosis, HE stained frozen sections from the Finnish samples subjected to microarray experiments were subjected to pathologist's review. All fibroids were histologically classified as typical leiomyomas. All had a mitotic rate of less than 1 per 10 high-power fields, and the semi-quantitative assessment of cellularity classified them into 3 groups as follows: 22% in class 1, 73% in class 2, and 5% in class 3. A trend for greater cellularity in *FH*-mutant fibroids was observed, as none of them belonged to class 1, and the only tumour in class 3 was *FH*-mutant (p=0.101, Fishers's exact test).

6.2.2 Microsatellite analysis on 1q and FH mutation analysis (III)[2]

The microsatellite analysis was performed on the set of 166 fibroid and 51 corresponding normal myometrium samples. At the loci flanking the *FH* locus, 46 individuals (153 tumours) were informative at least with 1 of the 2 markers. Allelic imbalance was observed in 5 samples. Of the 6 Finnish HLRCC fibroids, 4 showed LOH on chromosome 1q indicating loss of the wild-type *FH*: The sporadic fibroids harbouring AI on 1q were subjected to mutation analysis of the FH gene; Two mutations were detected, a missense mutation c.586G>A (p.A196T) in sample 32m1, and a splice-site mutation IVS4 + 3A>G in sample 4m3. The corresponding normal myometrium and all other tumour samples from these patients were also subjected to *FH* mutation analysis, but no changes were detected. No second point mutation was detected either in the HLRCC fibroids.

6.2.3 7q LOH mapping (II)

The 166 sample set was also subjected to LOH mapping on chromosome 7q. All tumours were informative with the majority of 25 markers covering about 20 Mb on chromosome 7q21-q31, and 11 (8 different patients) showed allelic imbalance with at least 2 markers. Three individuals (9, 14, and 38) had 2 different tumours that showed LOH on 7q, in 2 cases (9 and 38) the lost alleles were the same, but in case 14 different chromosomes were lost in 2 different tumours of the same patient. In most of the cases, the regions of AI were consistent, and there were no regions where heterozygosity was retained in the middle of AI. Three tumours did not meet the criteria for LOH with all markers within the region of AI, but true retention of heterozygosity and thus the possibility of a homozygous deletion was ruled out by a clear trend towards LOH seen in the allele intensity curves. The microsatellite analysis defined a common region of AI flanked by markers 7qAC005070 on 7q22.3 and 7qAC007567 on 7q31.1 being compatible with a deletion of 3.2 Mb. This region harbours at least 22 different transcripts, 20 of which are represented on the HG-U133A chip by 31 different probe sets.

[2] The 1q LOH and *FH* mutation analysis of the set of 166 sporadic fibroids has been published by Lehtonen et al. (Lehtonen et al. 2004), and is not part of this thesis work. However, since the methods and samples are the same, and as the identification of these sporadic *FH* mutant fibroids was crucial for the expression microarray study of this thesis, the information is presented here.

6.2.4 Microarray comparative genomic hybridisation (aCGH) (II, IV)

To further analyse the nature of the AI observed in the fibroids, aCGH experiments were performed. The first analysis was performed with a 1 Mb resolution aCGH chip of the whole genome. Ten out of the 11 fibroids with 7q LOH were successfully hybridised, and 8 of them showed deletion in the region predicted by LOH; two samples did not show any changes in the region of AI. On the other hand, one of them (51m1) demonstrated whole chromosome gains of chromosomes 8, 9, 12, 14, and 19. No homozygous deletions were observed. The same approach was also applied to the HLRCC fibroids from the UK; all showed LOH on chromosome 1q.

To further scrutiny the deletions on 7q, a tiling path aCGH focusing on chromosome 7q was produced and more fibroid samples from the UK were added to the experiment. Altogether 42 uterine leiomyoma samples were tested against a common female normal DNA; 20 of them showed deletion. The deletions were clustered around two different regions. The first was delimited proximally by clone RP11-437M1 in sample 38M1, and distally by clone RP11-443I10 in sample 34M1, with a length of 2.79Mbp, consistent with the microsatellite analysis. The second deletion was observed in 5 samples and was located between clones RP11-795E24 in sample 14M4, and RP11-786A19 in sample 29M2. No homozygous deletions were observed with the high-resolution array CGH.

6.2.5 Gene expression analysis (II, III)

A total of 33 samples from 12 patients were successfully hybridised on expression microarray chips (III). There were 11 samples of normal myometrium (N), and the 22 tumour samples consisted of 3 different subgroups: 9 fibroids with AI on chromosome 7q (7QD), 6 fibroids with no detected AI on 1q or 7q (N7Q), and 7 samples with mutations of the *FH* gene. A preliminary unsupervised hierarchical cluster tree (Figure 4) with all 33 samples was constructed using the 466 most differentially expressed genes within the samples set. The procedure divided the samples into 3 major branches, one mainly consisting of normal myometrium, one of fibroids carrying *FH* mutations, and one of *FH* wild-type samples, some of which carried 7q deletions. Also, technical issues did not seem to be a major cause of differential gene expression as the samples did not cluster according to the person who had prepared the samples or the day on which the samples were run (Figure 4).

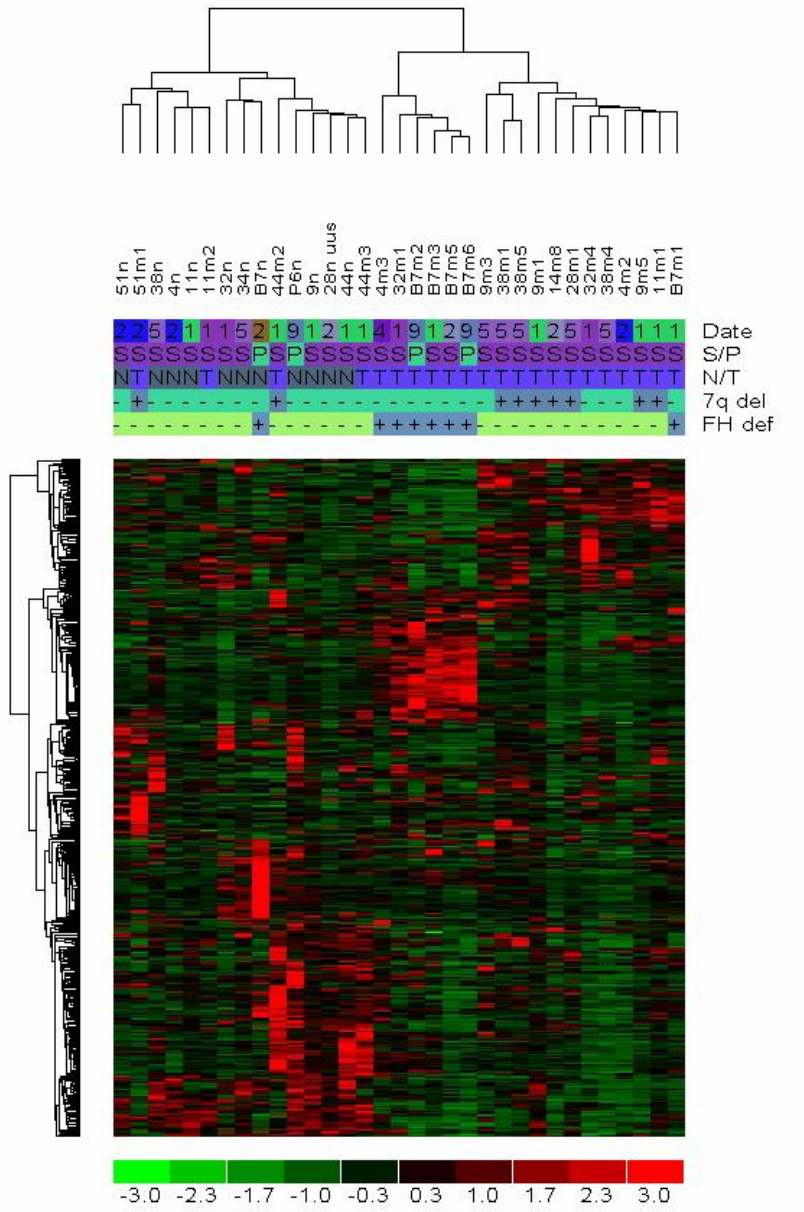

Figure 4. An unsupervised cluster tree of all samples. Date refers to the hybridisation batch; S/P to different person performing the hybridisation; N normal; T tumour; 7q del, samples showing AI on chromosome 7q; FH def, samples carrying *FH* mutations.

6.2.6 Fibroids compared to normal myometrium (II)

The first comparison of gene expression data was conducted with the *FH* wild-type and normal myometrium only. This allowed us to compare our own data with already published results on fibroid gene expression without the confusing effect of the apparently different *FH*-mutant fibroids. By comparing the groups 7QD and N7Q together to the normal myometrium samples, 133 differentially expressed probe sets were identified with a 5% rate of estimated false discoveries. Fifty four of them were up-regulated and 79 down-regulated in the fibroids. Of some genes, several

independent probe sets were available providing an internal control for the reproducibility of the expression data. For example, *FN1* was represented by 4 probe sets, the fold-changes of which ranged from 1.72 to 1.79, increasing our confidence in the data.

Although several interesting individual genes, such as *growth arrest and DNA-damage-inducible beta* (*GADD45B*), *retinoic acid receptor responder 3* (*RARRES3*), *homeo box A5* (*HOXA5*), *B-cell CLL/lymphoma 6* (*BCL6*), *death-associated protein kinase 1* (*DAPK1*), *cyclin D1* (*CCND1*), *phosphodiesterase 8B* (*PDE8B*), *transcription factor AP-2 gamma* (*TFAP2C*), and *protein kinase C beta 1* (*PRKCB1*), were observed to be deregulated in fibroids, the power of genome wide expression analysis lies in the simultaneous detection of changes in multiple functionally related genes, that is the pathways. Gene Ontology (GO) annotations were utilised to group the genes into biologically meaningful categories. Almost 90% (87 out of 111) of the genes differentially expressed could be put into one or more categories and together they belonged to 515 different groups. A functional group enrichment test showed that 40 of the ontology terms represented by more than two genes were enriched with genes differentially expressed (unadjusted p-value < 0.05). However, several of them were overlapping or nearly identical, and thus the true number of differentially expressed functional groups was substantially lower. For example, genes related to transcription, 4 of which were up-regulated (*SATB2*, *SOX4*, *TFAP2C*, *TRPS1*) and 13 down-regulated (*ATF3*, *BCL6*, *CITED2*, *EGR1*, *GATA2*, *HOXA5*, *JUN*, *KLF2*, *KLF4*, *MSX1*, *NFIB*, *NR3C1*, *NR4A2*) in fibroids, belonged to multiple different groups (Transcription Factor Activity, Transcription, Transcription Regulator Activity, and DNA Binding). Other categories with increased representation on the list of differentially expressed genes were such as Response to Stress with 13 genes, 3 of which were up-regulated (*DDB2*, *ERCC3*, *FN1*) and 8 down-regulated (*APOL3*, *BCL6*, *C1R*, *C1S*, *GADD45B*, *MAP3K5*, *NR3C1*, *NR4A2*, *PDLIM1*, *SMAD7*) and Extracellular Matrix with 9 genes up-regulated (*COL1A1*, *COL3A1*, *COL4A1*, *COL4A2*, *COL5A2*, *COL9A2*, *LTBP2*, *MFAP2*, *MMP11*) and 2 down-regulated (*LTBP1*, *TNXB*) in fibroids. If a conservative Bonferroni adjustment for multiple testing was applied, only 3 categories, all representing extracellular proteins (Extracellular Matrix Structural Constituent, Collagen, and Extracellular), reached a statistically significant confidence of enrichment.

6.2.6.1 Fibroids with 7q-deletions compared to fibroids without deletions (II)

The next objective was to compare the 2 groups of *FH* wild-type fibroids (7QD and N7Q) to each other in order to specify the global changes on the gene expression profile caused by chromosomal deletions of 7q. As already indicated in the unsupervised clustering of the fibroids, the changes between 7QD and N7Q fibroids were not very significant. In trying to identify the most differentially expressed genes, this was reflected by the large number of estimated false discoveries; even with the most stringent conditions, FDR was estimated to be >50%. A two-sided heteroscedastic t-test was utilised to identify the most likely genes affected by the 7q loss, and the 107 probe sets with a p-value of less than 0.01 were further analysed for enrichment in any particular GO category. Altogether, 22 categories with 2 or more genes had an unadjusted p-value less than 0.05, and the categories most clearly enriched were Nuclear Membrane, ER to Golgi Transport, Endomembrane System, and Nucleic Acid Binding, although none survived the Bonferroni correction for multiple testing.

Although the global expression pattern did not significantly differ between 7QD and N7Q, the possibility remained that the expression data could give some clues as to which gene within the commonly deleted region could function as a tumour suppressor. Therefore, the expression of the probe sets located between the clones AC005070 and AC007567 were evaluated in more detail. The 31 probes represented 20 of the 22 different transcripts in the region, and thus expression data was not obtained from 2. In addition, 6 probe sets were called absent in all 25 samples, 2 probe sets were present only in 1 sample and 1 probe set only in 2 samples. Also, one of the probe sets representing *PP35* was detected in 5 normal samples but only in 3 tumours. This left 13 transcripts out of 22 that were consistently expressed at detectable levels. Most of these genes showed marginal down-regulation in 7QD fibroids, although no single gene stood out as a striking candidate (II). Only two of them had an unadjusted p-value smaller than 0.05: *component of oligomeric golgi complex 5* (*COG5*, p = 0.02) and *Rad50-interacting protein 1* (*RINT-1*, p = 0.04). *COG5*, however, had also another probe set that did not show as clear a down-regulation.

6.2.6.2 Fumarate hydratase-deficient fibroids (III)

To test the hypothesis that *FH*-mutant fibroids form a distinct group among uterine fibroids, the expression profile of *FH*-mutant fibroids was compared to that of groups 7QD and N7Q in the Finnish set of samples. Firstly, a hierarchical clustering analysis of only the fibroids was performed. This separated the fibroids into two major branches, one of which comprised both sporadic *FH*-mutants, all but one HLRCC fibroid, and both fibroids from patient 44 (III). The samples were also separated according to their FH-status in the principal component analysis plot (III). When a FDR of 5% was allowed, 360 probe sets showed differential expression between *FH*-mutant and wild-type fibroids. They corresponded to 297 genes, 41 of which were represented by more than 1 probe set, showing consistent changes in gene expression. A GO annotation could be assigned for 214 of these genes, and they belonged to 1519 different categories. In the functional group enrichment test, 50 of them showed significance at the level of 1%. Genes belonging to Hexose Metabolism category were the most significantly enriched group within the differentially expressed genes ($p = 1.7$ E-09) and, for example, all 10 differentially expressed genes involved in Glycolysis ($p = 1.1$ E-08) had higher expression in *FH*-mutants. In fact, of the 50 categories, 14 were directly involved in carbohydrate metabolism, and all but a few of the genes, such as *FH*, had higher expression in *FH*-mutants. Additional categories with higher expression in *FH*-mutants included Iron Ion Homeostasis, Oxidoreductase Activity, Membrane Lipid Catabolism, Integral to Endoplasmic Reticulum Membrane, and Electron Transporter Activity. Genes with lower expression in *FH*-mutants, on the other hand, tended to belong to functional categories such as Extracellular Matrix, Cell Motility, Muscle Contraction, Organogenesis, Muscle Development, Cell Adhesion, and Plasma Membrane. When a conservative Bonferroni correction was applied, only 10 categories, all representing genes involved in carbohydrate metabolism, showed significant enrichment on the list of differentially expressed genes. In addition, known HIF target genes (Semenza 2003), not a GO category, showed significant enrichment within the differentially expressed genes ($p = 2.2$ E-08); of the 45 genes tested, 13 had higher expression in *FH*-mutant fibroids.

The expression profile of *FH*-mutant fibroids was also compared to the normal myometrium. The genes involved in glycolysis were again highly significantly over-

represented among on the list of differentially expressed genes (p = 1.3E-07), and all categories that survived the Bonferroni correction were related to carbohydrate metabolism. Also, many other genes showing higher expression in *FH*-mutant fibroids when compared to *FH* wild-type fibroids, such as *NQO1*, *AKR1C1*, *TKT*, *CDKN1A*, *ME1*, *PRDX1*, *PRDX4*, *PRDX6* and *LDHA*, showed higher expression when compared to normal myometrium.

The expression data from the UK fibroids facilitated the validation of our findings in an independent set of samples. To test the overall similarity of the Finnish and UK samples, an unsupervised clustering was performed; the UK HLRCC fibroids clustered together with the Finnish *FH*-mutants, and the UK sporadic fibroids clustered with the Finnish *FH* wildtypes (Figure 5). This gave confidence that the two sets of samples were, at least to some extent, comparable with each other. An unadjusted t-test was performed to find the genes most significantly differentially expressed between the UK normal myometrium and HLRCC samples. This confirmed the up-regulation of several genes involved in glycolysis, such as *LDHA* (FC 2.3, p = 0.04), *ENO1* (FC 2.5, p = 0.0006), *PKM2* (FC 2.0, p = 0.026), *PFKP* (FC 3.0, p = 0.01), and *PGAM1* (FC 1.6, p = 0.01). The up-regulation of *TKT* (FC 2.5, p = 4.8E-7), *NQO1* (FC 2.5, p = 0.005), and *CDKN1A* (FC 4.3, p = 0.016) was also confirmed. Indeed, most genes behaved in a similar way in the UK sample set, although statistical significance was not seen in all cases. There were, however, genes the deregulation of which could not be confirmed; *CDKN1C*, for example, was the sixth most significantly down-regulated gene in the Finnish sample set (FC 0.35, q = 0.0026),[3] but in the UK set the significant down-regulation was not observed (FC 0.9, p = 0.57).

A molecular predictor based on gene expression data was also built to test whether a small number of genes (<10) could be used to predict the *FH* status of fibroids. In the leave-one-out cross-validation performed on the Finnish set of samples, a predictor consisting of *LDHA*, *NQO1*, *LAMA2*, *BNIP3*, *MYO15B*, *CDKN1C*, and *COL6A2* gave the best results, and thus it was subsequently tested on the independent set of 7 UK fibroids. The 7-gene classifier correctly predicted the *FH* mutation status of all UK fibroids.

[3] The q-value is a modified p-value based on the estimated false discovery rate described by Tusher et al. (2001).

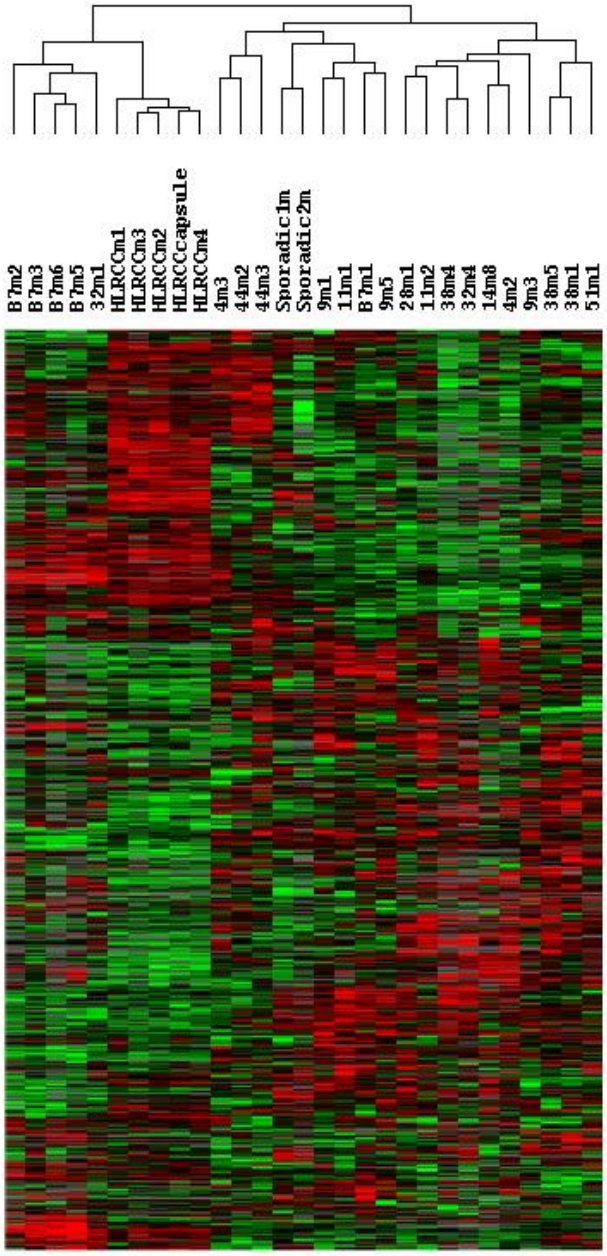

Figure 5. An unsupervised cluster tree of the Finnish and UK samples. UK *FH*-mutants (HLRCC1-4, capsule) clustered together with Finnish *FH*-mutants and UK FH wildtypes (Sporadic1m, 2m) clustered together with Finnsih *FH* wildtypes.

6.2.7 RT-PCR (II, III)

In order to validate some of the expression data with an independent method, quantitative real-time RT-PCR was performed on some of the genes. The down-regulation of *DAPK1* and *RARRES3* in *FH* wild-type fibroids compared to normal myometrium was confirmed as both showed significant down-regulation with p-values of 0.019 and 0.038, respectively. As marginally significant down-regulation of *RINT-1*, a gene within the commonly deleted 7q-region, was observed between 7QD

and N7Q fibroids, further expression analysis was performed, but no significant deregulation was detected (p = 0.75). A similar approach was applied to evaluate some of the genes defining the *FH*-mutant expression profile, the higher expression of *TKT* (p = 0.006), *LDHA* (p = 0.023), *CDKN1A* (p = 0.043) and *AATF* (p = 0.004) in *FH*-mutant fibroids compared to *FH* wildtypes was confirmed.

6.2.8 Western blotting (III)

As 10 out of 12 *FH*-mutant fibroids included in the expression analyses showed robust genetic evidence for loss of FH (all UK HLRCC fibroids showed 1q deletion, the Finnish sporadic *FH*-mutants and 3 out of 5 HLRCC tumours showed 1q LOH), in only 2 cases the loss of FH was not genetically confirmed. Also, although *FH* mutation negative, both fibroids from patient 44 clustered together with *FH*-mutant fibroids in the expression microarray analsis (III). Therefore, Western blot analysis was performed to evaluate the FH protein level in 7 samples, normal myometrium B7n and 44n, and fibroids B7m1 (no 1q LOH), B7m2 (showed 1q LOH), B7m3 (no 1q LOH), 44m2 and 44m3. Of the HLRCC fibroids, B7m1 had clearly the highest FH level, but no reduced expression of FH was observed in 44m2 or 44m3 (III).

6.2.9 7q gene screening (IV)

For the 7q candidate gene screening, the minimal deleted region was defined proximally by the clone RP11-437M1; the distal delimiting marker was D7S496 as reported by Mao et al. (1999). Within this region, 10 manually annotated known protein coding genes (Ashurst, et al., 2005) were identified. In addition, *DLD* was sequenced as it was deleted in most samples with del(7q) and as it encodes a subunit of α-ketoglutarate dehydrogenase, an enzyme of the Krebs cycle. The genes were amplified in 134 PCR fragments, thus altogether 1474 PCR fragments in 11 samples were analysed; the success rate was ~93.8%. Altogether, 13 sequence alterations were observed in 8 genes (IV). Most of them (8) were known SNPs and they were observed in frequencies compatible with known allele frequencies available in the Ensembl data base. Of the novel SNPs, 4 were synonymous; one non-synonymous change was observed in *RINT-1* (c.349A>T, p.S69C). It was present as a homozygous change in 2 out of 8 patients (3 of 11 fibroids). A set of 93 healthy controls were also screened for this novel SNP, the frequency of the novel allele was 16%.

7 DISCUSSION

The work described herein has tried to answer some of the questions that arose from the identification of the molecular background of HLRCC. The research has followed two main lines, one of which is based on role of *FH* in renal cell cancer (I). The second line of research has focused on the molecular biology of uterine fibroids (II, III, and IV).

7.1 Renal cell carcinomas

As early-onset RCC is a component of the HLRCC, it was hypothesised that there should be *FH*-mutation carriers among patients who have suffered from RCC at a young age. Thus, a computerised search in the Finnish cancer registry database was conducted. The RCC cases in the Finnish HLRCC families are of papillary type II histology, but as there had not been any previous studies showing that only this type of RCC is associated with HLRCC, all histological subtypes were included in the analysis. The search resulted in a list of 19 interesting families. Although the original hypothesis was that some of these patients should carry mutations in *FH*, the medical history of Family A (Figure 3), especially the cardiac paraganglioma in the index case's mother, suggested that SDH could be involved instead. Indeed, both the index case and his mother carried germline R27X mutation in the *SDHB* gene. Both tumours showed loss of the wild-type allele in the tumour sample, suggesting that *SDHB* acted as a tumour suppressor, an observation compatible with earlier studies on *SDHB*-related tumours.

This Finnish case of clear-cell RCC in the *SDHB*-paraganglioma syndrome was the first indication that malignant renal tumour might be a component of the syndrome. Interestingly, at the same time, studies in a population-based registry of the Freiburg-Warsaw-Columbus Phaeochromocytoma Study Group, which registers all clinical phaeochromocytoma cases diagnosed in Germany and central Poland, and at the time had 352 unrelated registrants, identified another family with an *SDHB* germline mutation c.847-50delTCTC in which 2 siblings had suffered from early-onset RCC with solid histology at ages 24 and 26, respectively (Family B, Figure 6). Both RCCs showed loss of the wild-type allele at the mutation locus suggesting that the function of *SDHB* had been abolished, and both siblings suffered from

paraganglioma as well. Together these studies strongly suggested that RCC did not occur in these patients by chance but truly belonged to *SDHB*-associated tumour predisposition. The mutation screening effort was extended to cover all 4 SHB-genes in a mixed set of 60 Finnish sporadic RCCs, but no mutations were detected. In addition, a separate set of 35 clear-cell RCCs diagnosed before the age of 50 were screened for all 4 genes in America with negative results.

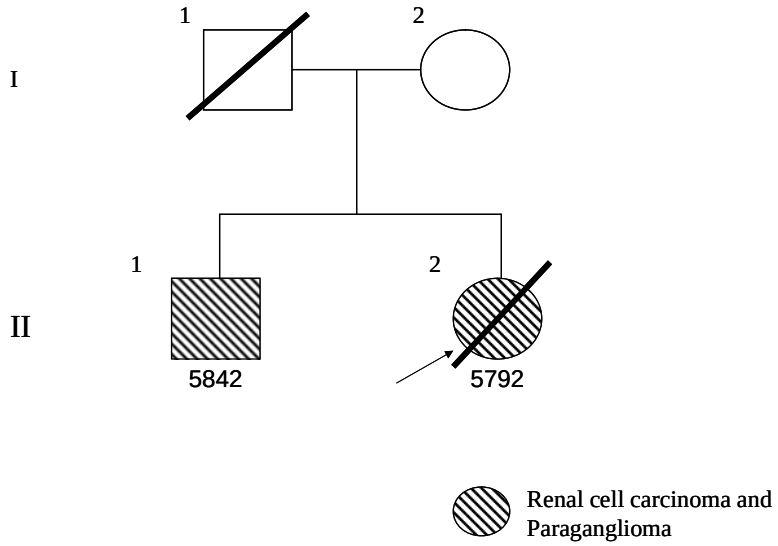

Figure 6. Family B ascertained from the Polish-German registry of phaeochromocytomas.

RCC provides a link between FH- and SDH-associated neoplasia. Although the histological types of these tumours are different, mostly papillary type II in HLRCC and clear-cell or solid histology in SDHB-associated tumours, all these tumours arise from the proximal tubule cells in the kidney. Both SDH- and FH-deficient cells have been shown to over-express HIF and its target genes (Isaacs et al. 2005, Selak et al. 2005). Also, HIF-stabilisation is important in VHL-associated clear-cell RCC development (Kim and Kaelin 2004), Tsc2-deficient RCCs over-express HIF2 and its target genes (Liu et al. 2003), and hepatocyte growth factor (HGF), which is the ligand for the MET receptor tyrosine kinase, triggers a signalling cascade that activates HIF1 (Tacchini et al. 2001). Thus it seems that the unifying theme behind most familial RCCs is HIF-signalling.

If this finding is further confirmed, it will establish a new form of hereditary RCC. Therefore, it has immediate clinical implications for the medical surveillance of both patients with early-onset RCC as well as familial paraganglioma or phaeochromocytoma. It is notable that patient II-1 in Family B (Figure 6) received his

diagnosis of RCC before the paraganglioma was diagnosed. Also, as a significant amount of apparently sporadic paraganglioma/phaeochromocytoma has undetected germline mutations in SDH-genes, the risk of RCC should be kept in mind also in the follow-up of these patients.

Thus far, only 3 cases of RCC have been detected in families with SDH-related paraganglioma. In the German-Polish registry, 2 cases of RCC were identified among 31 *SDHB* mutation carriers, but more cases could still develop as 40% of these individuals are younger than age 20. Therefore, the risk of RCC within *SDHB* mutation carriers could be estimated to be 5-10%. However, as both cases were diagnosed in the same family, it seems that all individulas do not have the same risk of RCC. This resembles HLRCC, in which there are clear differences in the risk of malignant phenotype between families (Kiuru and Launonen 2004). The possibility of a genetic modifier for the risk of RCC in HLRCC has been vigorously studied, although no candidates have been identified yet. Whether such a modifier exists, and whether the same modifier could affect the risk of RCC among HPGL patients also remains to be seen.

7.2 Uterine leiomyomas

The part of the study that was aimed at uterine fibroids was conducted to test 3 different hypothesis related to fibroid biology (see Hypotheses on page 38). Based on previous studies, recurring chromosomal rearrangement had been commonly observed on chromosome 7q. In this study we further scrutinised the commonly deleted 7q region and analysed whether the presence of 7q-deletions significantly affected fibroid gene expression profile. In addition, the known genes within the commonly deleted region were screened for mutations. Also, fibroids carrying mutations in the *FH* gene were compared to *FH* wild-type fibroids by gene expression profiling.

7.2.1 7q fibroids

The microsatellite analysis of chromosome 7q revealed a common 3.2 Mb region of allelic imbalance of within 7q22.3-q31.1, flanked by clones AC005070 and AC007567. This region of AI was observed in 11 out of 166 fibroids. No retention of heterozygosity was observed within the regions of AI, although some of the markers did not meet the criteria for AI in all samples. Also, in different samples, the allele intensity peak of the lost allele varied to some extent. Although it could only result

from the varying amounts of normal contaminating cells in the sample, the possibility that some tumours would be mosaics with regard to 7q AI could not be ruled out (Xing et al. 1997). When this result was combined to that of Mao et al. (1999), the smallest common region of AI was only 2.3 Mb.

Allelic imbalance, or LOH, is detected by assessing the relative abundance of maternal and paternal alleles at the site of a polymorphism. If a tumour sample is completely pure and no normal cells are present, heterozygosity is truly lost when all copies of a particular allele are derived from one of the parents. This, however, is seldom the case, as normal and tumour cells grow and interact together *in vivo*, therefore resulting in normal tissue contamination. LOH can occur either by a true loss of a particular allele, or by a mechanism that only changes the relative amount of alleles without any effect on gene dosage. The latter can be achieved at least by three different mechanisms (Gaasenbeek et al. 2006). Firstly, one chromosome homologue can be lost while the remaining homologue is duplicated (or one homologue is duplicated and then the second homologue is lost). This would result in whole chromosome loss. Secondly, LOH from a telomere and extending to a part or whole of the chromosome arm can result from break-induced replication in a process referred to as mitotic recombination. Thirdly, interstitial LOH can be observed after a process called gene conversion that occurs through a mechanism probably similar to mitotic recombination. Therefore, solely by analysing LOH it is not possible to detect chromosomal deletions, only AI.

An additional source of information that can help in understanding chromosomal rearrangements is comparative genomic hybridisation. While this method measures the abundance of DNA in comparisons to normal genome dosage, it can identify chromosomal copy number changes without the information on the relative number of different parental alleles. Thus, these two methods, LOH analysis and aCGH complement each other, and they have been used together successfully to analyse chromosomal rearrangements, for example in colorectal cell lines, in which the chromosome aberrations were found to be sometimes complex and explainable only with more than 2 independent genetic events (Gaasenbeek et al. 2006).

The Finnish fibroid samples showing AI on 7q were further analysed with aCGH to study the nature of AI. In addition, 35 UK samples from a set of 309 fibroids, 25 of which showed AI on 7q, were included in the aCGH analysis. This showed that the region of LOH was most often due to chromosomal deletions,

although some samples did not show copy number changes. No copy number changes were observed in samples showing no AI on 7q, and no homozygous deletions were detected. As commonly deleted chromosomal regions have been shown to harbour tumour suppressor genes, this finding was consistent with our hypothesis H2 (Gnarra et al. 1994). Homozygous deletions have been shown to harbour tumour suppressor genes (Devilee et al. 2001, Li et al. 1997) and therefore such deletions could have further narrowed our minimal deleted region. This was the first time the existence of small homozygous deletions on chromosome 7q has been studied in uterine fibroids, and it seems that at least they are not a common event in uterine fibroids. However, the presence of deletions smaller than 90 kb would not have been detected with the high resolution aCGH used, and therefore, small deletions may have escaped our analysis.

An expression analysis in which fibroids showing 7q LOH were compared to fibroids with no 7q LOH was performed, aiming at the detection of putative tumour suppressor genes as down-regulated genes. At the global level, 7QD and N7Q fibroids did not significantly differ from each other, as shown in the unsupervised clustering of the samples. The same was reflected in the fact that no significantly differentially expressed genes could be detected between the two groups. This suggested that 7q-deletions do not have a significant effect on fibroid gene expression profile, therefore contradicting hypothesis H3. Still, it might be that the deletions target an important gene or genes which contribute to the development of these tumours. Therefore, a more targeted analysis was performed to look at only the genes within the commonly deleted region. Although the expression of most genes within the minimal deleted region was detected, the analysis could not unequivocally identify a clear candidate gene. Most genes in this region showed down-regulation in 7QD fibroids, but statistically significant down-regulation was not observed.

The known protein coding genes within the commonly deleted region were screened for mutations, but no pathogenic changes were detected. This finding could be due to several reasons (Devilee et al. 2001). Firstly, it could mean that the initial hypothesis (H2) is wrong and that there is no leiomyoma suppressor within the commonly deleted region. Also, the tumour suppressor might not be a known gene, in which case further sequencing of the remaining putative genes within the region could prove fruitful. The mutations may not reside within the coding region of the putative suppressor gene and therefore the search for the mutations should have been

conducted within the regulatory regions of these genes. In addition, the tumour suppressor could lie outside of the region covered by mutation analysis, or there might be more than one leiomyoma suppressor on 7q. Evidence that the commonly deleted 7q region breaks down into several different regions has been provided in a recent study that detected a small 500 kb deleted region proximal to our minimal deleted region (Sell et al. 2005).

Recent reports have shown that *PBEF*, a gene within the commonly deleted region on 7q, functions as an insulin-like regulator of glucose homeostasis (Fukuhara et al. 2005), as well as a factor regulating arterial smooth muscle cell maturation and response to apoptosis (van der Veer et al. 2005). PBEF was shown to promote vascular smooth muscle cell differentiation through an NAD^+-dependent mechanism, and when *PBEF* was down-regulated by siRNA, the process was slowed down. Although these results were obtained with vascular smooth muscle cells, the *PBEF* could also have a role in uterine fibroid development. *PBEF* showed down-regulation in all fibroids compared to normal myometrium, but a difference between 7QD and N7Q fibroids was not detected. It is possible, however, that 7q-deletions are only one method of down-regulating *PBEF* and that the same effect could be achieved by other mechanisms, such as promoter hypermethylation, in N7Q fibroids. This has not, however, been studied. As the mutation analysis did not detect any point mutations in *PBEF*, there is no evidence of a second hit in the *PBEF* gene. Thus, *PBEF* could be haploinsufficient (Cook and McCaw 2000), and therefore only one hit could be enough to promote tumorigenesis. In this case, the mutation screening should have been targeted to the group in which no 7q-deletions were detected. The haploinsufficiency model cannot as easily be accommodated to cover tumours in which LOH is observed but that do not show deletion. If a gene would have a mutation, the duplication of the mutant chromosome would lead to complete loss of the functional gene whereas, if there were no mutation, the LOH would not have an effect at all. Therefore, a 50% decrease in the gene dosage could not be easily achieved. In the initial aCGH screen, 2 out 10 fibroids showed LOH but no deletion. One of these samples showed deletion in the tiling-path array, the other sample failed. These results indicate that the vast majority of 7q LOH in fibroids is caused by deletions and, thus, the haploinsufficiency model has to be kept in mind.

7.2.2 Fumarate hydratase-deficient fibroids

In a recent study by Lehtonen et al. (2004) the involvement of *FH* mutations in sporadic uterine fibroids was examined through microsatellite analysis of chromosome 1q and subsequent *FH* mutation screening. Out of 153 informative cases, 5 showed LOH and 2 of these carried mutations in the *FH* gene. None of the other fibroids from the same patients had *FH* mutations, nor were the mutations detected in the corresponding normal myometrium indicating that the mutations had been acquired some time during the fibroid formation, followed by clonal expansion of the mutant tumour cell population. The result indicated that *FH* is indeed involved in the formation of a subset of sporadic fibroids, and that as in most cases of HLRCC tumours, two independent hits are required for tumorigenesis. This further supported role of *FH* as a tumour suppressor gene, following the Knudson's two-hit hypothesis. The frequency of *FH* alterations in sporadic fibroids has been studied by other groups too (Barker et al. 2002, and 2006, Gross et al. 2004). Both studies by Baker et al. resulted in negative results, but Gross et al. concluded that karyotypic alterations of the *FH* locus might indeed play a role in sporadic fibroids. Taken together, the evidence supports the role of *FH* in a small subset of sporadic fibroids. As no sporadic mutations have been detected in studies performed on samples from other populations than Finnish, it could be that there are some modifying genetic or other factors that could favour the tumorigenesis to follow the pathway that involves loss of FH. A similar pattern of incidence has been observed regarding uterine leiomyosarcoma, as 3 Finnish HLRCC families have cases of leiomyosarcoma, a tumour type that has not been detected in families from other populations (Kiuru and Launonen 2004). A recent study indeed shows that Finnish HLRCC patients seem to have an increased risk for developing leiomyosarcoma (Lehtonen et al. 2005), although somatic mutations in *FH* are rare in these tumours (Ylisaukko-Oja et al. 2006).

To test hypothesis H4, according to which FH-deficient fibroids form a distinct subclass of fibroids, expression microarray analysis was conducted. Already the unsupervised clustering of all samples suggested that *FH*-mutant fibroids, both sporadic and HLRCC, were significantly different from their *FH* wild-type counterparts. The *FH*-mutant fibroids formed their own cluster and, furthermore, principal component analysis separated the samples into distinct groups (III), except for sample B7m1, which tended to cluster with *FH* wild-type fibroids. This raised the

question whether this sample would actually not be FH-deficient and thus represent a non-syndromic fibroid in a syndromic patient. Western blot analysis confirmed that in contrast to B7m3, another fibroid that did not have a second genetic hit on *FH*, B7m1 expressed FH protein. Therefore, the global gene expression analysis of fibroids suggested that *FH*-mutants, regardless of their origin, have a distinct expression profile and, for this profile, two hits in *FH* are required. Two hits have indeed been detected in most HLRCC tumours (Kiuru and Launonen 2004).

As already indicated by the clear differences at the global gene expression level, a number of significantly differentially expressed genes between *FH*-mutants and wildtypes were detected. A gene ontology (GO) based approach for mining functional groups in the set of differentially expressed genes found that most significantly enriched gene categories were related to carbohydrate metabolism. Most of these genes were up-regulated in *FH*-mutant fibroids. Other categories over-represented among the genes differentially expressed were such as Oxidoreductase activity, Iron Ion Homeostasis, and Extracellular. The lower expression of extracellular matrix proteins was also reflected in the fibroid histology as *FH*-mutants tended to have a higher level of cellularity than *FH* wildtypes. In addition to these GO categories, genes known to be activated by HIF (Semenza 2003) were over-represented within the genes differentially expressed, most showing higher expression in *FH*-mutants. The HIF-signature was well concordant with findings by others who have shown increased microvessel density in fibroids from HLRCC patients (Pollard et al. 2005). Also, recent findings have clearly linked TCA cycle defects with HIF1α stabilisation (Isaacs et al. 2005, Selak et al. 2005), and a gene expression study on phaeochromocytomas of various genetic backgrounds found induction HIF target genes in VHL- and SDH-defective tumours when compared to MEN2- and NF1-related tumours (Dahia et al. 2005).

It is of interest that glycolytic genes were so strikingly up-regulated in *FH*-mutant fibroids. The glycolytic phenotype is a well known feature of cancers (Gatenby and Gillies 2004), and increased flux of glucose in cancer cells was already observed by Warburg (1956). Also, glycolytic enzymes such as phosphoglycerate mutase (PGM) and glucosephosphate isomerase (GPI) that were found to be up-regulated in *FH*-mutant fibroids (III) can also modulate cellular life span (Kondoh et al. 2005). As HLRCC patients seem to have an increased risk of leiomyosarcoma (Lehtonen et al. 2005), it might suggest that the increased expression of glycolytic

genes in *FH*-mutant fibroid could lead to increased malignant potential. If this were the case, *FH* mutations should also be detected in sporadic leiomyosarcomas, which seems to be rare (Ylisaukko-Oja et al. 2006), however.

7.2.3 Reading fibroid gene expression profiles

Often the approach to tumour gene expression profiles focuses on the differences between the lesions, aiming at defining all molecular subtypes of a particular tumour type (for example Perou et al. 2000). This would then lead to the identification of specific pathways that drive each subclass, and therefore the subclass information would have clinical implications (Sorlie et al. 2001). There are examples where genetic dissection of a particular tumour type has lead to significant results, such as the identification of *epidermal growth factor receptor* (*EGFR*) mutations in lung cancer as a factor determining response to a specific protein kinase inhibitor called gefitinib (Stratton and Futreal 2004).

An alternative approach to genome-wide gene expression data could be the identification of the common pathways deregulated in several tumour types (Rhodes et al. 2004). If the focus is only on the differences, the things that are common, potentially the key pathways that actually drive the tumorigenesis of the particular tumour type, may be ignored. Recent evidence suggests that the unifying theme in familial phaeochromocytoma development is resistance to a particular apoptotic signal during development (Lee et al. 2005). In particular, Lee et al. have shown that mutations in *VHL*, *SDH*, and *RET* interfere with the c-Jun dependent apoptotic pathway so that it is not appropriately activated when nerve growth factor (NGF) is withdrawn; increased survival of *NF1*-mutant neuronal cells in the absence of NGF had been shown previously (Vogel et al. 1995). Lee et al. therefore proposed that the development of phaeochromocytoma in these familial syndromes was due to inappropriate developmental culling in some specific time-window during the development. Still, by gene expression profiling, these tumours could be divided into two different groups, possibly with a significant contribution from HIF-signalling (Dahia et al. 2005). Even if the fundamental mechanism of tumorigenesis in different classes of pheaochromocytoma is the same, the possibility remains that the activation of pseudo-hypoxic signalling due to *VHL*- and *SDH*-mutations in some of these tumours would promote the growth of these tumours significantly.

These two alternative approaches on genome-wide expression data also lead to at least two different models for fibroid development that take into account the existing molecular data (Figure 7). According to this distinction, the genes differentially expressed can be divided into two classes of interest, namely those equally differentially expressed in both *FH* wild-type and mutant fibroids, and those the expression of which is specifically altered in *FH*-mutants. Although in this study a tendency for higher cellularity in *FH*-mutants was observed, and others have identified distinct histological features in these tumours (Pollard et al. 2005), the *FH*-mutant fibroid histology is very similar to that of *FH* wildtypes. This suggests that the molecular pathobiology behind these lesions is also similar. Therefore, the genes that are commonly deregulated in both classes of tumours could tell us something about the essential gene expression changes leading to leiomyoma formation. On the other hand, since *FH*-mutants seem to have a clearly distinct expression profile, gene expression changes specific to these lesions could tell us something about the pathobiology specifically related to *FH* mutations. As discussed above, a clear HIF-signature was observed in *FH*-mutant tumours (III), a difference that is very similar to the differences observed between different types of phaeochromocytomas (Dahia et al. 2005). Therefore, the gene expression data obtained in our study is consistent with Model A in Figure 7, according to which all fibroids follow the same fundamental leiomyomagenesis pathway (the yet unidentified 'common fibroid pathway') with an additional HIF-signature that could lead to increased cellularity (III), activation of the glycolytic genes (III), and higher microvessel density (Pollard et al. 2005) in these tumours. Also, that no significantly differentially expressed genes could be identified between 7QD and N7Q fibroids supports this model (II). Alternatively, the fact that *FH*-mutant fibroids had a significantly different gene expression profile from *FH* wild-type fibroids could be interpreted as to support Model B in Figure 7. In this case, the focus should be put on the genes differentially expressed between these two classes of fibroids. In either case, when designing further studies, the problem of making testable hypotheses based on the identification of the lists of differentially expressed genes and the interpretation of them in functionally meaningful groups, must be faced.

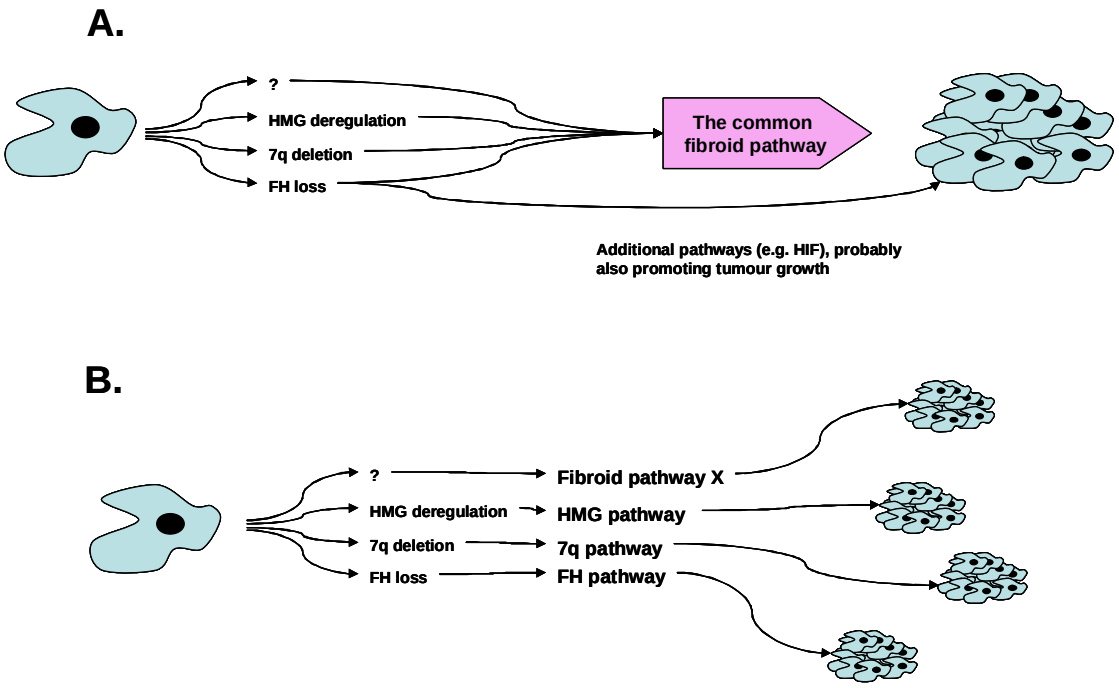

Figure 7. Two models for leiomyomagenesis. In A, each genetic defect leads to the activation of the common fibroid pathway which drives the tumorigenesis. Additional pathways may also contribute to leiomyomagenesis, but they are not the key pathways driving the pathogenesis. In B, each genetic lesion activates a pathway that is unique to that particular lesion resulting in biologically distinct subgroups of fibroids. A combination of these 2 models is also possible.

The derivation of meaningful predictions from the lists of differentially expressed genes has proven difficult. Several approaches have been developed, mostly relying on Gene Ontology annotations (Khatri and Draghici 2005), to analyse lists of genes in groups instead of only looking at individual genes. In some cases, the identification of a previously identified coregulated group of genes within the list of differentially expressed genes can be relatively easily interpreted. An example of this is the up-regulation of glycolysis related genes in *FH*-mutant fibroids (III). Previous experiments had already shown that HIF1 regulates the expression of these genes (Semenza et al. 1994), leading to a relatively easily experimentally testable hypothesis regarding *FH*-mutant fibroid biology. In this case, others have published experimental evidence that the down-regulation of either FH or SDH leads to the stabilisation of HIF1α and subsequent up-regulation of HIF1 target genes (Isaacs et al. 2005, Selak et al. 2005). Therefore the observation that glycolysis genes were up-regulated in *FH*-mutant fibroids could be interpreted as a signature of the HIF1 signalling, and in fact, the group comprising known HIF target genes was also significantly enriched within the differentially expressed genes (III). Often, however, it is hard to interpret a

relative over-representation of a particular gene ontology category within a list. For example, it is hard to formulate reasonably testable hypotheses from an observation that 9 out of 51 muscle contraction-related genes are enriched within the list of differentially expressed genes with a p-value of 0.0002 (III). Four of them were down-regulated and 5 up-regulated in *FH*-mutant fibroids when compared to *FH* wildtypes. Firstly, one could argue, that even if 9 of the genes are deregulated, there are still 42 genes the expression of which are not significantly altered. Can one, therefore, argue that the muscle contraction machinery is differentially expressed? Also, it might be that several of the genes involved in muscle contraction are expressed more than required for actual muscle contraction, and that only some of these genes have a function that regulate the amount of contraction. The question would then be, are some of these deregulated genes regulatory genes?

Even if the conclusion would be that the pathway is truly differentially expressed, it would still be hard to derive testable hypothesis from the observation. The deregulation of these kinds of gene sets that seem to have a common function is a vague observation and the causes leading to this apparently coordinated change in gene expression are hard to predict. Therefore, designing an experimental setting where one or more follow-up hypotheses could be tested may be extremely difficult. Especially, when the follow-up studies are typically very labour intensive, the evidence backing the predictions up should be as robust as possible.

If the use of gene groups based on similar function cannot be used in the further analysis of gene expression data, what would be the proper way forward? There are examples of studies where previous knowledge of functionally coregulated genes have been utilised in identifying some regulatory programmes in the existing data from gene expression profiling (Mootha et al. 2003a, and 2003b). For example, gene set enrichment analysis (GSEA) has identified coordinated changes of PGC-1α responsive genes in diabetic muscle (Mootha et al. 2003b). In addition, from previously published data, the same method was able to detect coordinated changes of genes from chromosomal regions commonly harbouring gross genetic alterations (Subramanian et al. 2005). Both of these observations, the activation of a transcription factor and a gross chromosomal deletion or amplification, can easily be put forward into hypotheses that can be experimentally tested. Therefore, it seems that a more direct link than merely a similar function or cellular location should be available between the different genes within a group before reasonable conclusions can be

drawn. To this end, several recent studies have brought new hope by trying to identify the regulatory regions of the human genome (Hallikas et al. 2006, Lim et al. 2005, Xie et al. 2005) and the combination of these kind of results with methods such as GSEA are likely to make the interpretation of microarray data significantly easier in the near future. Alternatively, a thorough understanding of the functional modules and regulatory circuits governing cellular processes, such as muscle contraction, might facilitate the prediction of the consequences of differential expression of individual genes. Such methods are being developed under the umbrella term 'systems biology' (Nurse 2003).

8 CONCLUSIONS

H1) A subset of early-onset renal cell carcinomas (RCC) is caused by HLRCC

With regard to H1, no definitive conclusions can be made. The fact that 19 out of 84 index cases had a family history indicating a possible hereditary syndrome, suggests that in early-onset RCC, familial predisposition could play a significant role. However, there is no evidence that any of these 19 cases would be caused by HLRCC.

One out of these 19 families was studied further, as the phenotype lead to an alternative hypothesis related to SDH. Subsequent experiments detected a truncating *SHDB* mutation in a cardiac paraganglioma and a clear-cell RCC, with some areas of eosinophilic staining. An additional family with paraganglioma and RCC segregating a germline *SDHB* mutation was also found in a Polish-German registry of phaeochromocytomas and paragangliomas. This was the first time extra-paraganglial tumours were detected in the *SDHB*-neoplasia syndrome, suggesting a novel form of hereditary RCC, and having immediate clinical implications for the follow-up of both patients with early-onset RCC and paraganglioma or phaeochromocytoma.

H2) Chromosome 7q21-31 harbours a leiomyoma suppressor gene

Several previous studies have indicated that 7q-deletions could play a role in leiomyomagenesis. We detected AI in 11 out of 166 fibroids from 51 patients, and further studies confirmed that AI was caused by chromosomal deletions. In addition,

in a separate set of UK samples, 8 fibroids with 7q AI were detected, all of which showed a deletion. With the high-resolution aCGH, the smallest commonly deleted region was 2.79 Mb, no homozygous deletions were observed. When new and previously published data were combined, the smallest common region of AI was delimited by the clone AC005070 and marker D7S496, covering only 2.3 Mb. The gene expression studies showed a subtle overall reduction in the expression of the genes within the common region of AI, but none of the genes stood out as a striking candidate suppressor. The 10 known genes within this region were sequenced for coding region and splice-site junction mutations in order to find a second hit. No mutations were detected.

The deletion mapping data is consistent with H2, as frequently deleted regions have been shown to harbour tumour suppressor genes. However, as no mutations in the known protein-coding genes were detected, it is not possible to implicate any of the genes within the common region of AI in leiomyomagenesis. Also, the data is not sufficient to exclude any of these genes from the list of putative suppressor candidates. Thus, the data does not strongly corroborate nor reject H2.

H3) Uterine leiomyomas carrying a 7q-deletion form a biologically distinct subgroup of leiomyomas

The global gene expression profiles of fibroids with and without 7q AI were studied by oligonucleotide microarray chips. These two groups did not separate from each other with unsupervised methods, indicating that the global expression pattern does not differ significantly. Similarly, no statistically significantly differentially expressed genes could be identified between these two groups. Thus, the microarray data indicates that 7q-deletions do not have a major impact on the fibroid gene expression profile, and therefore it may be postulated that these two groups of fibroids are biologically relatively similar. The data does not support H3.

H4) Fumarate hydratase-deficient uterine leiomyomas form a biologically distinct subgroup of leiomyomas

A global gene expression analysis of *FH*-mutant and wild-type fibroid was performed. A clear separation of these groups could be observed with unsupervised methods,

indicating that *FH*-mutant fibroids, both sporadic and HRLCC, have a distinct gene expression profile. In addition, the data suggests that two hits in *FH* are required for this distinctive expression profile to develop. Several significantly differentially expressed genes could be identified between these two groups, and they tended to be involved in carbohydrate metabolism. Also, a significant induction of hypoxia-inducible factor target genes was detected in *FH*-mutant fibroids. Based on gene expression data, *FH*-mutant fibroids have distinct biological properties that separate them from fibroids with wild-type *FH*, thus supporting H4. However, as the mechanisms driving fibroid initiation and progression are not known, based on this data alone it is not possible to tell if these mechanisms are different in *FH* wild-type and mutant fibroids.

9 EPILOGUE

The research described herein has attempted to answer some of the questions that arose after the clinical identification of the syndrome HLRCC and its molecular background. As is the case with most scientific discoveries, the detection of *FH* mutations in HLRCC families lead to a large number of new problems, new questions. Again, by trying to answer them, new questions arise. The results from this project have provided answers to a minority of the questions, but still, a large number of new questions have arisen. Is there a unifying mechanism behind the FH- and SDH-related neoplasia? Why are the tumour spectra different between these two tumour predisposition syndromes? Is the driving mechanism similar behind all uterine leiomyomas? Does the difference in fibroid expression profiles mean that the biological mechanisms behind different fibroids are truly different? If not, what role does the HIF-signature play in HLRCC fibroids? Which of the differentially expressed genes drive the leiomyomagenesis? Can the different expression profiles be explained by activation or inactivation of specific transcription factors? Thus, the initial clinical observation has lead to an exponentially expanding sequence of new scientific problems. By solving these problems we will hopefully be able to provide new ways to help the people suffering from one of the most devastating diseases, cancer.

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